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Ultrastructure and Chemical Composition of Microconidial Walls of Trichophyton Mentagrophytes

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ULTRASTRUCTURE AND CHEMICAL COMPOSITION OF
MICROCONIDIAL WALLS OF TRICHOPHYTON MENTAGROPHYTES

by

Christine Da-Ruh Wu

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

June

1976

DEDICATION

**TO MY PARENTS, IN TESTIMONY OF MY GRATITUDE FOR
THEIR LOVE, GUIDANCE AND SACRIFICE.**

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BIOGRAPHY

Christine Da-Ruh Wu was born on December 5, 1948 in Shang-hai, China. She graduated from the First Girls' High School in Taipei, Taiwan in 1966 and entered the National Taiwan University in Taipei, Taiwan the same year. She graduated with a Bachelor of Science degree, with a major in Botany.

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LIST OF ABBREVIATIONS

A	angstrom
C	centigrade
cm	centimeter
F	fraction
x g	gravity
g	gram
hr	hour
kv	kilovolt
μg	microgram
μl	microliter
μm	micrometer
mA	milliampere
mg	milligram
ml	milliliter
min	minute
M	molarity
nm	nanometer
N	normality
%	percent
R	residue
SDS	sodium dodecyl sulfate
sec	second
UMS	8 M urea-1 % mercaptoethanol- 1 % SDS in 0.1 M sodium phosphate buffer (pH 6.5)
w/v	weight per volume

CHAPTER I

INTRODUCTION

The microscopic morphology of fungi depends largely upon the arrangement of components in their walls. Fungal walls possess considerable rigidity and thus provide the shapes of vegetative hyphae and various reproductive structures. Over the years, their structures and chemical compositions have been the subject of numerous reviews (Aronson, 1965; Bartnicki-Garcia, 1968; Gorin and Spencer, 1968; and Taylor and Cammeron, 1973).

Early investigators who studied the chemical and structural nature of the fungal wall generally subjected the whole cells to more or less drastic treatments in order to prepare a cell wall residue free from cytoplasmic contamination (Norman and Peterson, 1932; Blank, 1953; and Houwink et al., 1951). However, besides causing extensive chemical degradation or rupture of linkages of certain wall polymers, these drastic treatments often removed some cell wall components which were the integral parts of the walls. With the development of more gentle methods to effect cell breakage, it became possible to obtain chemically intact, clean fungal walls. Since then, considerable progress has been made in the areas of composition and structure (Crook and Johnston, 1962; Aronson, 1965; Bartnicki-Garcia, 1966; and Gorin and Spencer, 1968), taxonomy (Bartnicki-Garcia, 1970), differentiation (Bartnicki-Garcia and McMurrough, 1971), and biosynthesis (Phaff, 1971) of fungal walls. By means of polarization optics, X-ray diffraction

electron microscopy, and biochemical analysis, it has been possible to gain some insight into the organization of fungal walls at a level not revealed by light microscopy. Among the early studies concerning fungal wall morphology, Frey-Wyssling and Mühlethaler (1950) were the first to observe the fibrous structure in cell walls of Phycomyces. Their findings were further confirmed by the work of Roelofsen (1951) and Middlebrook and Preston (1952). Since then, such observation has been extended to include many other fungi. Houwink et al. (1951) demonstrated a fibrous texture in the chemically treated walls of yeasts, and subsequently, several of the aquatic Phycomycetes were investigated by Aronson and Preston (1960), leading to the demonstration of a fibrous wall structure in all cases. Besides the fibrous nature of the fungal walls, several electron microscopic studies revealed the multilaminated nature of the wall (Frey-Wyssling and Mühlethaler, 1950; Aronson and Preston, 1960). Ultrathin sections of cells of Saccharomyces prepared by Agar and Douglas (1955) revealed a multilaminate wall structure which appeared especially prominent in the bud scar regions. Further studies by Mundkur (1960) and Vitols et al. (1961) demonstrated a stratification in these walls indicating the complex and heterogenous nature of fungal walls. It is generally thought that the fungal wall is a characteristic two-phase system in which one phase, consisting of the microfibrils, is embedded within the other phase, the amorphous matrix (Aronson, 1965; and Hawker, 1965).

Although ultrastructural studies of fungal wall morphology have been enhanced by the increased resolution of the electron microscope,

less work has been reported concerning the ultrastructure of the spore walls. This is due partially to the technical difficulties often encountered in fixation and embedding of fungal spores.

Hawker and Abott (1963) and Necas et al. (1963) examined the sporangiospores of Rhizopus sexualis and R. nigricans by electron microscopy and reported a thick spore wall surrounded by black amorphous material. In Schizophyllum commune, the basidiospore wall was demonstrated to be made up of one fibrous layer approximately 730 Å wide (Voelz and Niederpruem, 1964). The dictyospore of Alternaria brassicicola was shown to have a two layered wall; the outer layer distinct from the protoplast being melanized and electron dense, and an inner electron transparent layer (Campbell, 1969). Those of Ulocladium atrum were reported to be composed of an outer thinner wall of high electron density, and an inner fibrous wall of light electron density (Caroll and Caroll, 1974). Similar structures were observed in conidial walls of Botrytis cinerea (Buckley et al., 1966). Dormant basidiospores of Psilocybe possessed a thick wall composed of three layers, each of which differed in electron density from the other layers (Stocks and Hess, 1970). The outer layer was a fibrous layer and was continuous around the entire spore. It varied in texture from a thin hairy-like layer to a covering of long, loose fibers. The middle layer was a thick electron-dense layer of constant thickness except for the germ pore area, where it became a very thin covering. The inner layer was more electron transparent than the middle layer and was about 1/4 as thick. The thickness of the inner layer was

constant except for the areas around the germ pore and the apiculus.

In thin sections of Dictyostelium discoideum spores (Hemmes et al., 1972), three distinct layers were also distinguished. The outermost layer was highly electron dense and approximately 30 nm thick. The middle layer, which comprised the bulk of the spore coat, measured approximately 150 nm in thickness and was of light electron density. The innermost layer, adjoining the plasma membrane, was about 30 nm thick and of moderate electron density. Also, conidiospores of Penicillium and Aspergillus species usually possessed a three-layered wall. Ghiorse and Edwards (1973) studied the ultrastructure of conidial walls of Aspergillus fumigatis. They reported an electron dense outer layer, or a thin "cuticle" (10-20 nm), covering the young conidia which upon maturation, developed rapidly into a thick dense "cuticle" (25-180 nm in width) characteristic of matured conidia. The innermost wall layer also was of relatively high electron density and overlaid the plasma membrane. Between the cuticle and the innermost layer was a zone of medium electron density, probably composed of chitinous material. These structures were reported in other Aspergillus species such as A. nidulans (Florance et al., 1972), and A. niger (Tsukahara et al., 1966).

Spores having more complicated walls, as many as four or five distinct layers have also been reported. Walls of Tilletia caries teliospores consisted of four distinct layers; two outer wall layers, a partition layer, and an inner layer. The outer and inner wall layers appeared to have a fibrous texture, while the middle layer which gave

the reticular surface pattern, appeared to be homogeneous. The relatively thin partition layer separated the two outer walls from the inner wall (Allen et al., 1971). Of the various fungal spore walls examined, ascospores of Neurospora tetrasperma appeared to have the most complex wall structure (Lowry and Sussman, 1968). Light microscopy showed that it was composed of three major layers including an inner layer, the endosporium; a middle layer, the episporium; and an outer layer, the perisporium. Observations with the electron microscope revealed the presence of two additional layers. One of these was relatively electron transparent and covered the electron dense ridges of the perisporium. It could be sharply distinguished from the perisporium itself, which was electron dense and chemically distinct. The second additional layer revealed by the electron microscope was composed of a very electron-dense material lying between the episporium and the endosporium. Most of the time, it was found to be associated with the electron transparent endosporium.

Other structural studies carried out on spore wall morphology included those on ascospores of Saccharomyces cerevisiae (Hashimoto et al., 1958; and Beckett et al., 1973); blastospore walls of Candida albicans (Montes et al., 1965; and Cassone et al., 1973); conidiospore walls of Penicillium griseofulvum (Fletcher, 1971), and Blastomyces dermatitidis (Garrison and Lane, 1974); chlamydospore walls of Candida albicans (Miller et al., 1974); meiospore walls of Allomyces species (Olson, 1974); sporangiospore walls of Pseudoperonospora cubensis and Phytophthora infestans (Cohen et al., 1974) and Linderina

pennispora (Young, 1971); uredospore walls of wheat stem rust (Williams and Ledingham, 1964); and zoospore walls of Klebsormidium flaccidium (Marchant et al., 1973), and Phytophthora erythroseptica (Vujicic et al., 1965).

The surface structure and ornamentation of fungal spore walls have recently attracted attention of several workers. By using carbon replicas, chlamydospore wall of Tilletia caries (Swinburne and Matthews, 1963), conidiospore walls of Penicillium (Hess et al., 1968), spore walls of fleshy fungi (Bigelow and Rowley, 1968), Ustilaginales (Zogy and Schwinn, 1971), and a few pathogenic fungi (Smith and Sandler, 1971) have been examined. Recently, the use of freeze-etching techniques have provided better resolution than replica techniques for the study of surface configuration of fungal spores. The unique fracturing process yielded views of internal and surface structures of the walls without chemical fixation that were not possible with ordinary thin sectioning methods. Sassen et al. (1967) examined the conidiospore walls of Penicillium megasporum using both thin sectioning and freeze-etching techniques and found that these walls could be divided into several different layers. The surface of the spores as seen with freeze-etched preparations appeared to be covered with small "rodlets" arranged in a characteristic manner and varying in length between 50 and 5000 Å. A base layer could also be seen. In thin sections, a separate layer of dense material approximately 90 Å thick was assumed to correspond to both the "rodlets" and the base layer. Three layers of spore walls were observed, with the outer and

middle layers constituting approximately 80% of the total. The first layer (W_1) of spore walls lay directly beneath the base layer and in freeze-etched preparations consisted of a rough granular material. In chemically fixed sections, this layer appeared as an irregularly electron dense area and seemed to merge gradually into the next region, the W_2 layer. The W_2 layer was characteristically electron transparent in thin sections, but appeared to contain fibrillar material arranged parallel to the plasma membrane and was embedded in a finely granular matrix. The fibrils were thought to consist of chitin. The W_3 layer separated the fibrillar W_2 layer from the plasma membrane. It appeared as a slightly electron dense layer in thin sections, and was typically non-fibrillar and similar to the W_1 layer when examined with the freeze-etching techniques. This constructed the non-fibrous matrix in which the W_2 layer was embedded. Hess and Stocks (1967) also reported the observation of "rodlets" on the conidial surface of Aspergillus species using the thin sectioning and freeze-etching techniques. Similar structures have been observed on wall surfaces of basidiospores of Lycoperdon perlatum (Hess et al., 1972), and conidiospores of Oidiodendron truncatum and Gonotobotryum opiculatum (Cole, 1973). No rodlet structure has been reported on the freeze-etched surface of other spores, including basidiospores of Psilocybe (Stocks and Hess, 1970), Schizophyllum commune (Voelz and Niederpreum, 1964), and Panaeolus campanulatus (L) Fr. (Griffiths, 1971); ascospores of Aspergillus nidulans var. echinulatus and Chaetomium gelasinosporum (Hess et al., 1972); and spores of Dictyostelium

discoideum (Hemmes et al., 1972). The three dimensional structure of spore surfaces has been studied with the scanning electron microscope. Spores studied in this way include ascospores of Neurospora (Austin and Fredrick, 1974), Nannizzia and Arthroderma (Padhye, 1972), and conidiospores of Stemphylium botryosum (Corlett, 1973), and Microsporium gypseum (Visset, 1972).

Previous studies on the chemical composition of the fungal walls dealt with those of yeasts or the filamentous fungi (Crook and Johnston, 1962; Aronson and Machlis, 1959; Horikoshi and Arima, 1962; Bartnicki-Garcia and Reyes, 1968). In most cases, analyses were restricted to only the hyphal stage in the developmental cycle. Less attention was given to the localization of the individual polymers within the walls of fungal spores. Earlier studies on spore wall chemistry included those of Blackwell (1943) on oospores of Phytophthora species, Graham (1960) on teliospores of Tilletia, and Owens et al (1958) on ascospores of Neurospora sitophila. Subsequent studies reported were those of conidial spores of Deuteromycetes (Esquerre-Tugayé and Touze, 1971), ascospores of Lipomyces (Slooff, 1969), chlamydospores of Candida albicans (Janson and Nickerson, 1970), and basidiospores of Schizophyllum commune (Aitken and Niederpreum, 1970).

With the advent of combined biochemical and electron microscopical techniques, the structural constituents of fungal walls have been investigated by subjecting walls to series of chemical extractions or enzymatic digestions. Mahadevan and Mahadkar (1970) subjected the

walls of Neurospora crassa to a series of alkali and acid extractions and chemically analyzed the individual fractions thus obtained. The major constituents of the conidial wall were identified as protein-polysaccharide complex, (1→3)- β -glucan, and chitin, which were quantitatively similar to those reported in the mycelial walls. In Dictyostelium discoideum spores, three layers were revealed by electron microscopic studies (Hemmes et al., 1972). Hemmes and coworkers degraded the spore coat with the enzymes cellulase and pronase and reported that the outer layer was composed of mucopolysaccharides, the middle layer of cellulose; and the innermost layer of cellulose and protein.

It is well known that fungal spores, upon germination, give rise to their vegetative counterpart, the vegetative hyphae. Despite the morphological dissimilarity between hyphal and spore walls, their chemical compositions have been reported to be qualitatively similar. Bartnicki-Garcia and Reyes (1964) studied the chemical composition of sporangiospore walls of Mucor rouxii and compared them with that of the vegetative forms. No qualitative, but only quantitative, differences in wall compositions were observed. Horikoshi and Iida (1964), by acid hydrolysis and enzyme digestion, found that the conidial walls of Aspergillus oryzae resemble their hyphal walls in chemical composition. However, a lower chitin and higher protein content were observed in the conidial walls. When similar studies were performed on Penicillium chrysogenum (Rizza and Kornfeld, 1969), the conidial walls were found to be richer in galactose and contained a higher

concentration and greater variety of amino acids than did the hyphal walls. Martin et al. (1973) divided the germination process of Penicillium notatum conidia into four morphologically distinct stages (resting spores, swollen spores, germinating spores, and mycelium), and observed the changes in chemical composition that occurred in the spore walls. They found that the conidial and mycelial walls were chemically different in the distribution of amino acids, neutral sugars, and amino sugars. Galactose was the most abundant sugar in walls of resting spores, while glucose was the main component of hyphal walls. The content of the amino sugars galactosamine and glucosamine also increased greatly during germination. Similar studies were also carried out on dimorphic fungi. Chattaway et al. (1968) analyzed the wall composition of mycelial and blastospore forms of Candida albicans, and found that the former contained three times as much chitin as that from the blastospore wall and only about one third as much protein. When sporangial and cyst walls of Phytophthora palmivora were analyzed (Tokunaga and Bartnicki-Garcia, 1971b), they were found to consist of mainly (90%) β -glucan with 1,3-, 1,4-, and 1,6-linkages. However, relative proportions of the three differently linked residues were found to vary significantly between the two types of walls. Recent studies on chemical composition of oospore walls of Phytophthora megasperma var. sojae by Lippman et al. (1974) indicated the presence of a large amount of β -glucans (80%). Less than 10% were cellulose. Minor amounts of mannan and glucosamine were present. These values were comparable to those in the vegetative or sporangial walls of related species of Pythium (Bartnicki-Garcia, 1966; Sietsma et al.,

1975; and Tokunaga and Bartnicki-Garcia, 1971b).

Since most of the previous work concerning the chemical composition of fungal walls has been restricted to the hyphal stage in the developmental cycle, it is fruitful to review the literature on those subjects which are applicable to the study of the spore wall chemistry.

A. Polysaccharides.

Fungal wall polysaccharides are built from a variety of sugars. Among the 12 monosaccharides reported, D-glucose, N-acetyl-D-glucosamine, and D-mannose are the three most consistently detected constituent sugars of fungal wall polysaccharide. Those less frequently found, but yet characteristically distributed among certain fungi include: D-galactose and D-galactosamine (Ascomycetes), L-fucose (Mucorales and Basidiomycetes), D-glucosamine (Mucorales), D-xylose (Basidiomycetes), D-glucuronic acid (Mucor rouxii). The presence of minor amounts of rhamnose, ribose, and arabinose has also been reported in certain species of fungi (Bartnicki-Garcia, 1968). According to their component sugars, and the predominant configuration and position of the glycosidic linkages (or, in the case of heteropolymers, the structure of the main chain), the fungal polysaccharides can be grouped into several types as follows:

Chitin. Chitin, an insoluble unbranched polymer consisting of β -1,4-linked N-acetyl glucosamine residues, has been identified as a structural component of many fungal walls.

Schmidt (1936) was the first to detect chitin in certain filamentous yeasts such as Endomycopsis capsularis, and Eremascus fertilis. Roelofsen and Hoette (1951) subsequently detected, microchemically, the presence of chitin in 30 species of non-filamentous yeasts. Kreger (1954), using the x-ray diffraction technique, confirmed these findings and discovered that the chitin content in filamentous yeast was much greater than that of the budding yeasts, whose walls contain only 1 to 2 percent chitin. Eddy (1958), using an enzymatic approach, treated walls of Saccharomyces with chitinase and demonstrated the release of N-acetyl glucosamine. Similar work carried out on the yeast-like organisms included those on Cryptococcus (Jones and Farmer, 1967), Candida albicans (Chattaway et al., 1968), Saccharomycopsis (Buecher, 1968), and others (Phaff, 1971).

Chitin has been identified in walls of most filamentous fungi such as Phycomyces (Frey, 1950; and Aronson and Machlis, 1959); Aspergillus (Horikoshi and Arima, 1962), Neurospora (Blumenthal and Roseman, 1957), Penicillium (Kreger, 1954, and Troy and Koffler, 1969), Fusarium solani and Allomyces (Aronson and Machlis, 1959), Rhizidiomyces (Fuller and Barshad, 1960), and many others (Bartnicki-Garcia, 1968).

Like those methods employed in yeasts, the detection of chitin usually involved microchemical stain, x-ray diffraction of the acid and alkali resistant portion of the cell wall, and enzymatic hydrolysis of isolated polysaccharides. A higher level of chitin was observed in the filamentous fungi than in the yeasts, though the amount varied from species to species. Blumenthal and Roseman (1957) estimated quantitatively the chitin content in 25 strains of fungi and found them to vary

from 2.6 % (Neurospora crassa) to 26.2 % (Aspergillus parasiticus), while Aronson and Machlis (1959) reported the cell walls of Fusarium and Allomyces actually contained 47 % and 60 % chitin, respectively.

Although the physical nature of chitin in yeast walls is only partially understood, it has long been recognized as having an important role in maintaining the mechanical strength of hyphal walls of filamentous fungi (Bartnicki-Garcia, 1968). It is also the main constituent that gives rise to the characteristic fibrillar inner surface of fungal walls as revealed by electron microscopic studies (Roelofsen, 1951; and Aronson and Preston, 1960), and its dissolution was observed only upon drastic hydrolysis.

Chitin content was found to increase slowly with age when fungi were grown in surface culture in an acidic medium. An accelerated rate of chitin accumulation was observed when aeration was applied to the culture (Blumenthal and Roseman, 1957). Several workers have attempted to examine the relationship between morphology and alteration in chitin content of the walls. Katz and Rosenberger (1970) studied a mutant of Aspergillus nidulans that was defective in chitin synthesis. They found that these hyphae, though they contained low levels of chitin, still maintained their characteristic cylindrical shape and formed normal branches, provided the concentration of stabilizer was sufficiently high. Mahadevan and Tatum (1965), by examining the wall composition of wild type and several colonial mutants of Neurospora crassa, attempted to correlate the levels of structural polymers of the cell wall with wild type and colonial morphology. They observed that in some of the mutants, the chitin level was somewhat less than

in the corresponding wild types. However, in spite of this lower level, growth was normal in all cultures tested. Insofar as the role of chitin is concerned, there is no apparent consistent relationship between chitin level and morphology.

Cellulose. Although chitin is the most commonly encountered component of filamentous fungal walls, the presence of cellulose (β -1,4-linked glucan) as a minor component in certain chitin-deficient Phycomycetes and Oomycetes is well established. As with chitin, the most reliable characterization of cellulose is by x-ray diffraction. Frey (1950) reported the presence of cellulose in cell walls of Lagenidium, Saprolegnia, Achyla, and Phytophthora. Bartnicki-Garcia (1966) used x-ray diffraction and enzymatic studies and confirmed that cellulose, occurred in cell walls of Phytophthora cinnamomi, Phytophthora cactorum, Pythium debaryanum, Atkinsiella dubia, and Achlya ambisexualis. Microchemically, cellulose has been detected in a number of Oomycetous fungi (Nabel, 1939), Trichoderma lignorum, and spore walls of Dictyostelium discoideum (Ward and Wright, 1965). Although most cellulose containing fungi are chitin-deficient, some are reported to contain chitin. These include Mucor rouxii, Rhizidiomyces bivellatus, Fusarium culmorum, a Rhizidiomyces species, and Ceratocystis ulmi (Gorin and Spencer, 1968).

Glucan. Polysaccharides distinct from cellulose but composed of glucose residues are of common occurrence in fungal walls. The most thoroughly studied is yeast glucan, which constitutes a complete layer of cell wall of Saccharomyces cerevisiae, and properties of which have

been reviewed by Phaff (1971).

In nearly all of the early studies, yeast glucan was isolated after drastic treatment of whole yeast with hot dilute alkali followed by heating with dilute HCl for several hours. Bell and Northcote (1950) were the first, on the basis of methylation analysis, to obtain evidence for a highly branched polysaccharide of high molecular weight. Their evidence indicated a branched (1→3)- β -glucan with branched β -1,2-glucosidic inter-chain linkages. Further work done by Peat et al. (1958) suggested that the glucan was a linear molecule in which β -1,3, and β -1,6-linkages occurred at random or in sequences. Work by Misaki et al (1968) resulted in a return to the notion of a highly branched glucan, and they postulated a structure based on a "back bone" of β -1,6-linked glucose residues, to which were attached linear chains containing about eight β -1, 3-linked glucose residues. However, Manners and Masson (1969) have shown that the alkali insoluble glucan from Saccharomyces cerevisiae was actually heterogenous. It contained about 85 % of an insoluble branched (1→3)- β -glucan and about 15 % of a soluble branched (1→6)- β -glucan. Recently, similar results were obtained from glucan preparations from walls of the yeast Kloeckera apiculata, Schizosaccharomyces pombe, Saccharomyces fragilis, and S. fermenti (Manners et al., 1974). A similar type of glucan with a high proportion (63-73 %) of unsubstituted β -1,6-linkages was also described in Candida albicans and C. parapsilosis by Yu et al. (1967). In filamentous fungi, glucans with predominantly β -1,3-linkages were reported for Polyporus tumulosus (Angyal et al., 1974), Neurospora

crassa (Mahadevan and Tatum, 1965), Schizosaccharomyces commune (Wessels et al., 1972), Phytophthora (Bartnicki-Garcia, 1966), Aspergillus nidulans (Zonneveld, 1971), Oomycetes (Faro, 1972), Pythium (Sietsma et al., 1975), and others (Bartnicki-Garcia, 1968).

It has been known for a long time that (1 \rightarrow 3)- β -D-glucan might occur in microcrystalline conditions and form microfibrils. Recently Jelsma and Kregar (1975) reported that the conformation for (1 \rightarrow 3)- β -D-glucan was very similar to that proposed for (1 \rightarrow 3)- β -D-xylan based on x-ray fiber patterns. They also reported that the x-ray fiber pattern of the acid hydrolyzed fungal wall glucan was better crystallized. However, the acid treatment caused ultrastructural changes in the ultrastructural appearance of the glucan fibers. Electron microscopic examination of shadowed fragments of the treated walls revealed the presence of aggregates of short and spindle like, as well as microfibrillar, particles. Such structures were not observed to be present in the untreated walls.

A different glucan was isolated from walls of Schizosaccharomyces octosporus, S. pombe, and S. versatilis by Kregar (1954). He found that this microcrystalline glucan was alkali soluble and had an x-ray diffraction pattern different from that of yeast glucan. Kregar also identified this glucan in several other fungi. It was not until later that the structure of this glucan was identified. Studies carried out by Bacon et al. (1968) showed that it consisted of an α -1,3-linked glucose polymer, which also existed as the major component in the cell walls of Cryptococcus terreus and Candida albicans.

The α -1,3-glucan appears to be fairly widespread among higher fungi such as Basidiomycetes or Ascomycetes. In Aspergillus niger, two soluble glucans were detected by Johnston (1965); one of them was further soluble in hot water and was identified as nigeran (myco-dextran). The main component of this fraction was a glucan composed chiefly of α -1,3 and a small amount of α -1,4-linkages. Chemical analysis of hyphal walls of Schizophyllum commune by Wessels et al. (1972) have demonstrated the presence of an alkali soluble glucan (S-glucan) consisting of a straight chain of α -1,3-linked glucose residues. Moreno et al. (1969) found that the yeast phase of the dimorphic fungus, Paracoccidioides brasiliensis contained almost exclusively alkali-soluble glucan, while the mycelial form of this fungus contained only 60 to 65 % of this glucan. Angyal et al (1974) also reported the occurrence of such a glucan in cell walls of Polyporus tumulosus.

Mannans. Saccharomyces cerevisiae has long been known to contain mannans. Peat et al. (1958) subjected the pure yeast mannan to linkage analysis, and concluded that the backbone of the highly branched molecule contained α -1,6-linkages. Lee and Ballou (1965) were able to show the branched side chain consisted mainly of α -1,2 and α -1,3 linked residues. Mannans from yeasts other than Saccharomyces cerevisiae have also been reported in S. carlsbergensis, S. rouxii and Kloeckera brevis (Steward and Ballou, 1968), and Hansenula holstii (San Blas and Cunningham, 1974). In the mannan of the pathogen Candida albicans, the major antigen of the cell wall has been studied extensively due to

its immunological importance. Yu et al. (1967) studied the mannans of C. albicans, C. stellatoidea, C. parapsilosis, and C. tropicalis, and found that they were similar in structure, although there were some variations from species to species.

Mannan-protein complexes have been reported to be a major constituent of yeast cell walls. They serve as structural elements, as a matrix for immobilization of cell wall enzymes, and as sites for surface recognition (Gander, 1974). Many common yeasts have a mannan like that of Saccharomyces cerevisiae, as described above. Some other yeasts have mannan that lack side chains and contain 1,3- and 1,6-linked residues in a linear array, called a "block" structure by Gorin and Spencer (1968).

Recent chemical and immunological analyses carried out by Lipke et al. (1974) on yeast mannans showed that mannans of Kloeckera brevis, Kluyveromyces lactis, and Candida parapsilosis had the Saccharomyces type structure, whereas the mannans from Pichia bispora and Hansenula wingei had structures similar to each other and to that of Hansenula polymorpha.

The presence of a small amount of phosphate in yeast mannans was reported (Northcote and Horne, 1952). Mill (1966) was able to isolate and identify mannose-6-phosphate as a product of acid hydrolysis of mannan from Saccharomyces cerevisiae, indicating that in baker's yeast at least the phosphate appeared to be limited to the 6-position of the terminal non-reducing mannose residue of the main chain, or to the 1,2- or 1,3-linked residues of the side chains.

Kocurek and Ballou (1969) later showed the presence of phosphate in ten other yeast species and found that the phosphate content ranged from 0.04 % (Saccharomyces lactus) to 4.4 % (Candida atmosphaerica). Although the exact function of phosphate in mannan polymers is not clear, Jones and Ballou (1969) suggested that the phosphate groups tend to inhibit the action of exo- α -mannosidase, thus preventing digestion of the mannan. Recently, a glucosyl phosphate residue in a yeast mannan was found to play an important role as an immunochemical determinant (Lipke et al., 1974).

Galactomannan. Not all of the mannans extracted with alkali from yeast cell walls contain mannose exclusively as the component sugars. Gorin and Spencer (1968) were able to isolate a galactomannan from Nadsonia and several species of Trichosporon. Structural analysis of the galactomannan from Trichosporon fermentans showed the ratio of mannose to galactose to be 65:35. Side chains terminating in a galactosyl residue were much more abundant than those ending in a mannosyl unit. They also investigated the galactomannan occurring in Candida lipolytica and found it to be very similar in structure to that of T. fermentans. Other yeasts in which Gorin et al. (1969) demonstrated galactomannans were Schizosaccharomyces, Torulopsis gropengiesseri, T. magnoliae and T. lactis-condesi.

The presence of galactomannan in certain filamentous fungi such as dermatophytes (Bishop et al., 1965), Aspergillus (Azuma et al., 1971) and Penicillium (Preston et al., 1970) has been reported (Gander, 1974). These heteropolysaccharides were usually associated with proteins or

peptides and formed a peptido-galactomannan complex which gave a hypersensitivity skin reaction. Azuma et al. (1971) purified and investigated the chemical and serological properties of galactomannans from Aspergillus flavus, A. effusus, Penicillium charlesii, P. notatum, P. frequentans and P. expansum. They concluded that galactomannan was a common antigen of Aspergillus and Penicillium species. Barker et al. (1963) isolated a peptidogalactomannan from dermatophyte mycelium, and analyzed the structure of the galactomannan. The predominant linkages were 1,2- and 1,4- to mannopyranosyl residues. The occurrence of 1,4-, and lack of 1,6-linkages, was unique among the polymers from dermatophytes. Other galactomannans investigated in dermatophytes include those from Trichophyton rubrum, T. granulosum, and Microsporum quink-eami (Gander, 1974).

Galactan. Galactose-containing polysaccharides are produced by a wide range of fungi. They occurred in walls of Penicillium griseofulvum, P. chrysogenum, Verticillium alboatrum, Mucor rouxii, and others (Gorin and Spencer, 1968). A heteropolymer containing up to 54 % galactose has also been reported in sorocarps of Dictyostelium discoideum, D. mucoroides and D. purpureum. However, they were reported to be absent from cell walls of Phytophthora cactorum, Saprolegnia ferax, Polystictus sanguineus, Mucor mucedo, Rhizopus stolonifer, and several yeasts.

Details of other minor fungal wall polysaccharides can be found in the review by Gorin and Spencer (1968).

B. Protein

Although not the major structural component, protein is usually detected in fungal walls. They were usually present as discrete surface layers (Bartnicki-Garcia, 1968; and Fisher and Richmond, 1970), as easily removable proteins, in the form of discrete internal protein layers (Manocha and Colvin, 1967), as glycoproteins that were firmly bound to the rest of the wall (Phaff, 1971; and Gander, 1974), or as enzymes associated with the walls (Bartnicki-Garcia, 1966; and Ruiz-Herrera, 1967).

Most of the studies carried out on fungal cell wall proteins have been limited to analysis of total amino acid composition (Eddy, 1958; Mahadevan and Tatum, 1965; Phaff, 1971; Roy and Landau, 1971; and Martin et al., 1973). Eddy (1958) was the first to report the presence of proteinaceous material in the cell walls of yeasts. Since then, a number of studies have been made of the kind of amino acids which occur in cell wall proteins of other yeasts. These included: Saccharomyces cerevisiae (Kessler and Nickerson, 1959), Trigonopsis variabilis (Senthe Shanmuganathan and Nickerson, 1962); Saccharomycopsis guttulata and Hanseniaspora uvarum (Shilfrine and Phaff, 1958). In filamentous fungi, a protein component present in Neurospora crassa was extensively studied. deTerra and Tatum (1963), and Mahadevan and Tatum (1965) reported its presence in this fungus, which was then isolated by Manocha and Colvin (1967) upon digestion of polysaccharide portions of the wall with hydrolytic enzymes. On the basis of electron microscopy, they concluded that this protein

material was a discrete layer within the wall and that it participated in determining the morphology of the wall.

In many cases, protein may often be found as a covalently linked complex with polysaccharide. Falcone and Nickerson (1956) and Korn and Northcote (1960) showed that yeast walls contained three protein-carbohydrate complexes: glucan-protein, mannan-protein I, and gluco-mannan-protein II. Extensive chemical analyses on structural configurations and glycosidic linkages of these molecules have been carried out (Phaff, 1971; and Gander, 1974).

Similar investigations have been conducted on cell walls of other yeasts, including various Hansenula species, Cladosporium werneckii, Candida albicans and Sporobolomyces species (Lloyd, 1970; Kolarova et al. 1973; and Gander, 1974).

The occurrence of several extracellular peptidopolysaccharides in filamentous fungi has been reported and their composition and function well elucidated (Gander, 1974). The peptidogalactomannans isolated from Trichophyton species (Barker et al., 1963) and Aspergillus fumigatus (Azuma et al., 1968) were considered as allergenic agents since they usually caused an immediate or delayed hypersensitivity reaction in man or guinea pigs. It was thought that these glycoproteins must be located sufficiently near the outer surface to serve as immunogenic agents. Although their functions were not known at the time, other fungal glycoproteins reported included the peptidopolysaccharide containing glycosyl, mannosyl, and galactosyl residues from Neurospora crassa (Wrathall and Tatum, 1973) and a peptidophospho-

galactomannan from Penicillium species (Gander, 1974).

A number of enzymes in yeasts and fungi are found external to the cytoplasmic membrane, and bound tightly or loosely to certain wall components, although the exact location and the forces holding them to the wall are not completely understood. Many of them usually have carbohydrate covalently attached to the peptide. Enzymes such as invertase, melibiase, glucamylase, α -amylase, inulinase, trehalase, acid phosphatase, catalase, and glucosidase have been found in cell wall of yeasts and yeast-like organisms (Phaff, 1971; and Kessler and Nickerson 1959). Of these enzymes, invertase (β -fructofuranosidase) was most extensively studied. It was found to be a glycoprotein containing 50 % mannosyl, and 30 % glucosamyl residues (Neuman and Lampen, 1967). Metzenberg (1963) purified and investigated a glyco-invertase from Neurospora crassa walls containing 11 % mannose and 30 % glucosamine. It was found to be covalently bound to a cell wall component and was not released upon enzyme digestion or certain chemical extraction (Trevithick and Metzenberg, 1966). Other cell wall associated enzymes isolated from fungi include galactofuranosidase from Penicillium species (Gander, 1974), the glucosamylase from Aspergillus niger (Pazur et al., 1963), acid phosphatase from Saccharomyces species and Candida albicans (Gander, 1974), and proteases from Saccharomyces carlsbergensis. (Maddox and Hough, 1970).

C. Lipid

The presence of lipids in fungal walls has been well documented (Bartnicki-Garcia, 1968). They usually existed as either bound or free

lipids, the latter being extractable by organic solvents without previous hydrolysis. It has been reported that contrary to cytoplasmic lipids, cell wall lipids lack palmitoleic acid and were mainly composed of saturated fatty acids (Bartnicki-Garcia, 1968). There is great divergence in the values reported for lipid in various fungal walls. Masschelein (1959) determined as much as 13.5 % lipid in walls of beer yeast after acid hydrolysis. Eddy (1958), on the other hand, found less than 2 % total lipid in walls of various Saccharomyces species. In sporangiophore walls of Phycomyces, lipid was mostly cuticular in nature and accounted for more than 25 % of the dry weight (Kreger, 1954). However, Aronson and Machlis (1959) were not able to detect lipids in isolated walls of Allomyces macrogynus. Since there was no assurance that the lipid rich cytoplasmic membrane completely removed during the washing and purification of walls, much of the reported variation in lipid content was probably due to the extent of removal of these membranes.

The role of lipid in fungal wall has not been well elucidated. Some workers believed that the firmly bound lipid might have a structural role. Hurst (1952) suggested that lipid contributes to the stiffness of the cell wall of Saccharomyces cerevisiae. Nickerson (1974) also cited evidence for a possible role for lipids in the architecture of yeast cell walls. In certain spores and sporangiophores, lipid was thought to contribute to their hydrophobic properties (Bartnicki-Garcia, 1968). In Hansenula ciferrii, the acetylated phytosphingosines found on the cell surfaces were thought to be

responsible for the tendency of such yeasts to form pellicles in liquid media and for the mat appearance of the colonies (Phaff, 1971).

D. Ash

Ash has been determined by incineration of walls, and its composition examined using spectrographic analysis. Elements such as P, Mg, Ca are the major constituents of the ash, while others like Si, Fe, Cu, Cr, Al, Ba, Co, Mn, and Na can be detected in lesser amounts.

In most bacterial and fungal walls, the amount of ash accounted for only 2 to 3 % of the dry weight of cell walls. In contrast, walls of some fungi such as Allomyces (Aronson and Machlis, 1959), yielded high quantities of ash (8-10 %), the nature of which was not determined. In Mucor rouxii, an even higher proportion of ash (16 %) was reported (Bartnicki-Garcia and Nickerson, 1962).

Although the nature of the wall components from which ash is derived was not clear, in some cases the presence of inorganic components was related to the chemical nature of the wall, which exhibited a binding capacity for certain ions. For example, in ascospore walls of Neurospora, 11 % of the spores total cations might be bound to the wall (Sussman et al., 1967). In Mucor rouxii, chitosan was thought to be involved in the binding of phosphate in their walls which were known to be composed of large amounts of chitosan phosphate salts (Bartnicki-Garcia and Nickerson, 1962).

E. Melanins

The presence of pigments similar to or identical with melanin has been reported in cell walls of certain species of fungi. However,

only a few detailed studies of fungal melanins have been reported. Emerson and Fox (1940) showed that the brown pigment of the outer walls of sporangia of Allomyces was probably a melanin-like substance. The epispore of ascospore walls of Neurospora tetrasperma was referred to as a melanized layer (Lowry and Sussman, 1958). In 1964, Nicolaus et al. reported the presence of the melanin pigments in spores of Ustilago maydis and hyphae of Capnodium nerii. They found that the compositions of both types of melanins were non-indolic in nature. Bartnicki-Garcia and Reyes (1964) reported that a brown pigment partially extracted from spore walls of Mucor rouxii by hot alkali or acid was melanin. The isolated pigment constituted 17.3 % of the initial wall dry weight and was insoluble in organic solvents and soluble in alkali. But unlike typical melanins, the extracted brown pigment of this fungus was soluble in acid. It contained 25.7 % of glucosamine, 14.8 % of protein, and an insignificant amount of anthrone positive carbohydrate. It was thought that the melanin could be associated with glucosamine in a triple complex with protein. Potgieter and Alexander (1966) analyzed hyphal walls of Rhizotonia solani and found that melanin-like materials accounted for 8.5 % of the wall weight. The presence of such a black pigment was also found in hyphal walls of Cladosporium species (Potgieter and Alexander, 1966). Chemical and elemental analyses confirmed that the pigment had characteristics typical of melanins. Bull (1970a) extracted melanin from hyphae of wild type Aspergillus nidulans and found that it accounted for 16.3 to 18 % of the wall dry weight. The pigment was insoluble in all non-

polar solvents and in mineral acids. However, hot alkali (0.5 N, 60 C, 24 hr) produced complete solubilization. The pigment appeared to be indolic in nature.

In spite of the occurrence of melanins in certain fungal species, the exact function of the pigment is still unclear at the moment. A positive correlation was demonstrated in several fungal species between the presence of such pigment and resistance to microbial or enzymatic lysis (Potgieter and Alexander, 1966). Lockwood (1960) noted that the dark hyphae of Helminthosporium sativum and Alternaria solani were more resistant to lysis than the hyaline hyphae. Bloomfield and Alexander (1967) reported that the melanin-covered sclerotia of Sclerotium rolfsii and the conidia of Aspergillus phoenicis were resistant to enzymatic digestion. However, the hyaline hyphae of both species were readily lysed. Similarly, the dark chlamydospores of Thielaviopsis basicola, in contrast with the albino spores, were resistant to lysis (Linderman and Tousson, 1966). In Aspergillus nidulans, evidence was also presented that the resistance of hyphae to lysis by a β -(1 \rightarrow 3) glucanase-chitinase mixture resulted from the presence of melanin in the walls (Kuo and Alexander, 1967). They also reported that the resistance of the walls to digestion was directly correlated with the melanin content of the mycelium. A melanin-less mutant was highly susceptible to hydrolysis by the enzyme mixture. Further studies undertaken by Bull (1970a) showed that this pigment was distributed throughout the hyphal wall of A. nidulans and was associated particularly with the chitin fraction.

A major problem in the analysis of the above data is the lack of uniformity in the methods used for degradation and analyses of the wall polymers. This is particularly evident in the analyses of wall polysaccharides.

Acid hydrolysis of polysaccharides to yield its constituent monosaccharides components is commonly used to study the carbohydrate composition of the fungal walls. However, as it is evident from the literature reviewed (Table 1), conditions chosen for hydrolysis have often differed from worker to worker. Acids usually employed for such purpose include sulfuric acid, hydrochloric acid, formic acid, nitric acid, trifluoroacetic acid, and others. Heating of wall material in acid is necessary, and as can be seen, it generally involved high temperature for various periods of time.

It has been reported that all monosaccharides are degraded to a certain extent by acid. For example, when T1 lipopolysaccharide of Salmonella friedenou was hydrolyzed with 0.5 N sulfuric acid at 100 C all of the D-ribose was liberated in 1 hr but only 28 % of the hexose and 20 % of the heptose were set free. After 12 hr, the latter two were obtained in quantitative yield, but 30 % of the D-ribose was decomposed (Berst et al., 1969). Similar results have been observed with a fructan (Lewis et al., 1967). Refluxing with 1 N sulfuric acid decomposed 66 % of the D-fructose, but did not affect D-glucose. Nozawa et al. (1969) studied the degradation of individual monosaccharides released during hydrolysis from the polysaccharides. They found that glucose in general was more acid stable than mannose and galac-

TABLE 1. Conditions used for acid hydrolysis of fungal walls.

Organism	Conditions for Acid Hydrolysis	Authors
<u>Allomyces macrogenus</u>	85 % formic acid, 100 C, 16 hr Then 2 N HCl, 100 C, 2 hr	Aronson and Machlis (1959)
Ascomycetes	90 % formic acid, 100 C, 15 hr	Crook and Johnston (1962)
<u>Aspergillus nidulans</u>	1 N H ₂ SO ₄ , 105 C, 18 hr	Bull (1970a)
" "	Concentrated H ₂ SO ₄ , 4 C, overnight dilute to 2 N, 4 hr	Katz and Rosenberger (1970)
" "	40 % H ₂ SO ₄ , 4 C, 18 hr dilute to 5 %, 100 C, 3 hr	Zonneveld (1971)
<u>A. niger</u>	70 % H ₂ SO ₄ , 0 C, overnight dilute to 2 N, 100 C, 4 hr	Johnston (1965)
<u>A. oryzae</u>	20 N H ₂ SO ₄ , room temperature, 24 hr dilute to 2 N, 105 C	Horikoshi and Iida (1964)
Basidiomycetes	90 % formic acid, 100 C, 15 hr	Crook and Johnston (1962)
<u>Botrytis cinerea</u>	90 % formic acid, 100 C, 15 hr	Crook and Johnston (1962)
<u>Candida albicans</u>	70 % H ₂ SO ₄ , 0 C, overnight dilute to 2 N, 100 C, 4 hr	Chattaway et al. (1968)
Dermatophytes	1 N H ₂ SO ₄ , 105 C, 24 hr	Shah and Knight (1968)
<u>Dictyostelium discoideum</u>	1 N H ₂ SO ₄ , 100 C, 6 hr	Yamada et al. (1974)

TABLE 1. (Contd.)

Organism	Conditions for Acid Hydrolysis	Authors
<u>Dictyostelium discoideum</u>	6 N H ₂ SO ₄ , 25 C, 3 hr dilute to 3 N, 95 C, 3 hr	Hemmes et al. (1972)
<u>Hansenula holstii</u>	3 N HCl, 100 C, 3 hr	SanBlas and Cunningham (1974)
<u>Mucor rouxii</u>	1 N H ₂ SO ₄ , 105 C, 6 hr	Bartnicki-Garcia and Nickerson (1962)
" "	1 N H ₂ SO ₄ , 105 C, 24 hr	Bartnicki-Garcia and Reyes (1964)
<u>Neurospora crassa</u>	3 N HCl, 100 C, 3 hr or 4 N H ₂ SO ₄ , 100 C, 4 hr	Mahadevan and Tatum (1965)
" " "	4 N H ₂ SO ₄ , 100 C, 4 hr	Mahadevan and Mahadka (1970)
<u>Paracoccidioides brasiliensis</u>	1 N HCl, 100-105 C, 7 hr	Moreno et al. (1969)
<u>Penicillium chrysogenum</u>	4 N H ₂ SO ₄ , 100 C, 4 hr	Rizza and Kornfeld (1969)
<u>P. expansum</u>	1 N H ₂ SO ₄ , 105 C, 12 hr	Fisher and Richmond (1970)
<u>P. notatum</u>	1 N H ₂ SO ₄ , 105 C, 8 hr	Martin et al. (1973)
<u>Philophora verrucosa</u>	36 N H ₂ SO ₄ , 50 C, 5 min dilute to 2 N, 100 C, 2.5 hr	Szaniszlo et al. (1972)
<u>Phycomyces</u> sp.	90 % formic acid, 100 C, 15 hr	Crook and Johnston (1962)
"	1 N HCl, 105 C, 6 hr	Novaes-Ledieu et.al. (1967)

TABLE 1 (Contd.)

Organism	Conditions for Acid Hydrolysis	Authors
<u>Phytophthora</u> sp.	1 N H ₂ SO ₄ , 105 C or 22.5 N H ₂ SO ₄ , 30 C, 3 hr dilute to 0.85 N, 97 C, 4 hr or 6 N HCl, 105 C, 8 hr	Bartnicki-Garcia (1966)
<u>Phytophthora palmivora</u>	22.5 N H ₂ SO ₄ , room temperature, 3 hr dilute to 0.85 N, 97 C, 2 hr	Tokunaga and Bartnicki-Garcia (1971b)
<u>P. megasperma</u> var <u>sojae</u>	22 N H ₂ SO ₄ , 25 C, 3 hr dilute to 0.85 N, 97 C, 4 hr	Lippman et al. (1974)
<u>Polyporus tumulosus</u>	72 % H ₂ SO ₄ , 4 C, 45 min dilute to 1 N, 100 C, 6 hr	Angyal et al. (1974)
<u>Schizophyllum commune</u>	0.5 N H ₂ SO ₄ , 100 C, 1.5 hr or 6 N HCl, 100 C, 6 hr	Wessels (1965)
" "	15 N H ₂ SO ₄ , 25 C, 48 hr dilute to 3 N, 121 C, 2 hr	Wang and Miles (1966)
" "	1 N H ₂ SO ₄ , 100 C, 6 hr	Wessels et al. (1972)
<u>Schizosaccharomyces pombe</u>	72 % H ₂ SO ₄ , 0 C, dilute to 8 %, 100 C, 4 hr	Bush et al. (1973)

tose. Galactose, when heated in 15 % methanolic HCl at 100 C for 15 hr, showed a 62 % loss. Noguchi et al. (1974) examined the rate of decomposition of hexosamine during hydrolysis using various concentrations of HCl for different periods of time. They reported that significant loss of hexosamine (35 % glucosamine) occurred when heated in 6 N HCl at 100 C for 24 hr, while a shorter period of incubation (4 hr) was optimal. It is generally agreed that hydrochloric acid causes more degradation than sulfuric acid (De Bruyne and Wouters-Leysen, 1971).

In addition to degradation of sugars, acids may convert sugars into anhydrous derivatives or cause isomerization to take place. Hydrolysis of polysaccharide in sulfuric acid often requires prior dissolution in concentrated acid (72 %) before heating in dilute acid at high temperature (Adams, 1965). However, it has been shown that part of the carbohydrate may become sulfated by this procedure, leading to erroneous results. The condition for acid hydrolysis of wall polysaccharides must, therefore, be carefully chosen and controlled for each biological specimen under specific conditions.

Current status of the morphology and chemical composition of the dermatophyte spores:

Earlier ultrastructural studies of dermatophytes have been reported in Epidermophyton (Laden and Erickerson, 1958; and Werner et al. 1964), Microsporum (Meinhof, 1966; and Werner et al., 1968), and Trichophyton (Meinhof, 1967; Ito et al., 1967; and Hasegawa, 1975).

However, little information is available regarding the ultrastructural morphology and chemical composition of the dermatophyte spores.

Among the very few morphological studies of the dermatophyte spores reported, most of them involved direct examinations of the spore surfaces by means of light or electron microscopy. In 1953, Ajello examined the rough macroconidial wall of the Microsporum species by means of light microscopy. A similar approach was followed by Stockdale (1963) in studying the rough surfaces of macroconidial wall of Microsporum species. Smith and Sandler (1971) were the first to take advantage of the scanning electron microscope for the observation of the dermatophyte spores. The surfaces of M. vucemosum macroconidium and Trichophyton erinacei arthrospore were examined. Subsequently, Akin and Michaels (1972) combined both scanning and transmission electron microscopy, and examined the ultramorphology and rough surface of macroconidial walls of M. gypseum. In ultra-thin sections, the wall was trilaminar in nature. It consisted of an outer electron dense layer; a fibrous, semi-transparent middle layer; and a semi-transparent innermost layer. The roughness of the macroconidial wall, as demonstrated by scanning electron microscopy, was reported to be due to the rounded projections which they termed "polyps". These were found to be continuous with, and of similar electron density to, the outer wall layer. Recently, ascosporeogenesis of M. gypseum was studied by means of electron microscopy, but little attention was paid to the ultrastructural morphology of their walls.

In spite of the recent investigations of the polymers constituting the hyphal walls of dermatophytes (Blank, 1953; Cummins and Harris, 1958; Shah and Knight, 1968; and Nozawa et al., 1973), our knowledge of the chemical and biochemical nature of dermatophyte spore walls has been very limited. This could be due to difficulties involved in purification and isolation of the spores and the preparation of their walls. Up to now, none of the pure spore walls of dermatophytes have been analyzed and chemically characterized. Recently, Page and Stock (1974) isolated the spore coat of M. gypseum by means of mechanical disintegration. These walls, along with those of the vegetative hyphae, were extracted with ethylenediamine for the isolation of glycoproteins. Two types of glycoproteins were reported: water-soluble and water-insoluble. The former accounted for 10 % of the dormant spore coat, but only 4 to 5 % of the vegetative wall dry weight. The latter which accounted for 3 to 3.5 % of both types of walls, were similar in amino acid composition. The water-soluble glycoproteins were rich in proline, threonine, glycine and glutamic acid; while the water-insoluble ones were rich in leucine rather than in proline. Besides the above mentioned data, no chemical analysis has been carried out on the isolated spore coat of this dermatophyte.

In Trichophyton mentagrophytes, the abundantly formed microconidia are thought to be of fundamental importance in the dissemination and survival of this dermatophyte. However, no information is available regarding the ultra-architecture and chemical nature of the microconidial walls. It is therefore pertinent to review some of the

knowledge available on the structural and chemical composition of hyphal walls of I. mentagrophytes. This information, though not directly related to the present study, does provide valuable information which can serve as a comparison for the data obtained in this study.

Previous ultrastructural studies of the vegetative walls of I. mentagrophytes were undertaken by Tsukahara et al. (1964). By means of electron microscopy, these investigators examined the ultrathin sections prepared from the vegetative hyphae. Besides the conventional fixation of specimens with osmium tetroxide, they found that several heavy metal stains were good fixatives for the walls. Staining after sectioning especially enhanced the contrast of the walls when examined under the electron microscope. When stained in a saturated solution of lead citrate or 2 % solution of uranyl acetate, after osmium tetroxide fixation, the walls appeared to be almost structureless, and had a thickness of 120 to 370 nm. Both the inner and outer regions of the wall were composed of thin electron-dense materials which surrounded the middle layer of less electron density. Subsequently, Pock-Steen and Kobayasi (1970) also studied the ultrastructure of the hyphal walls of I. mentagrophytes by means of thin sectioning. They demonstrated the double-layered structure of the walls. The outer layer was thin and electron dense, while the inner layer was thicker and electron lucid. The latter had a slightly fibrillar appearance, especially on the lithium permanganate fixed cells. Recently, the ultrastructure of I. mentagrophytes hyphal walls was studied after the use of a cytochemical reaction for polysaccharides. (Poulain et al., 1975). Electron

microscopic examination of ultrathin sections showed a wall composed of three layers; an external, poorly reactive one with a microfibrillar structure; a middle, fairly reactive one, with a periodic structure; and an internal, intensively stained layer, not observed in old cells.

Recently, by means of mechanical disruption, Noguchi et al. (1971) were able to isolate purified hyphal walls of this fungus and carried out chemical and structural studies. The electron microscopy of the shadowed wall preparations revealed a fibrous texture. The inner and outer surfaces were clearly different. The fibrils were very distinct on the inner surface of the wall. Chemical analysis indicated that they were composed mainly of a carbohydrate polymer (78.3 %), half of which was glucose (36.2 %), and a glucosamine polymer (30.4 %). Other neutral sugars detected were mannose (11.7 %) and trace amounts of galactose (less than 1 %). The peptide content of the walls was estimated to be 7.8 % with aspartic acid (11.6 %) and leucine (14.9 %) being the major amino acids. Other minor components were lipid (6.6 %), ash (2.2 %), and phosphorus (0.1 %). More recently, Kitajima et al. (1972) had attempted to clarify the localization and orientation of polymers in hyphal walls of I. mentagrophytes by electron microscopic and chemical studies. In a recent report, Noguchi et al. (1975) reinvestigated the neutral sugar composition of hyphal walls of this dermatophyte by means of gas liquid chromatography, and showed the presence of glucose and mannose at a ratio of 2.3:1. However, no galactose was detected.

In our previous work (Hashimoto et al., 1972), we were able to isolate and purify microconidia of T. mentagrophytes free from vegetative cells.

The purpose of this dissertation has been to elucidate the ultra architecture and chemical nature of the wall layers of T. mentagrophytes microconidia with particular emphasis on the localization of various components within the walls. To achieve this goal, the following studies were undertaken: 1) the determination of optimal conditions for various chemical analyses and acid hydrolysis of the microconidial walls. Since acid hydrolysis was frequently used throughout this study, the choice of specific acid, its concentration and the time of hydrolysis was critical. 2) the isolation and characterization of the microconidial walls. Microconidial walls were isolated in a pure state free from cytoplasmic contamination. Their ultrastructural morphology was examined by the electron microscopy, and their chemical composition was determined by analysis. For comparison, hyphal walls were also isolated and characterized chemically. 3) the chemical extraction and fractionation of microconidial walls. The isolated walls were subjected to several sequential and selective chemical extractions (alone or in suitable combinations) and/or enzymatic degradation. Each fractionation procedure was examined in regard to the soluble products and the insoluble wall residue obtained. Chemical analyses were carried out for each fraction, and the modification of the ultra-structural morphology induced by such treatments was examined by means of the electron microscopy (using such techniques as shadowing,

ultrathin sectioning, freeze-etching, and carbon-replica), and finally 4) preparation of a theoretical model of the conidial wall on the basis of the data obtained.

CHAPTER II

MATERIALS AND METHODS

Organism. Trichophyton mentagrophytes ATCC 26323*, obtained from Dr. J. Rippon of the University of Chicago, was used throughout this study.

Maintenance of the organism. Stock cultures were maintained at room temperature on Sabouraud dextrose agar (4 % glucose, 1 % neopeptone, and 1.5 % Bacto agar, Difco) with periodical transfer, about once a month, of the granular type colonies to prevent the pleomorphic transformation of the fungus.

Preparation of microconidia. Sabouraud dextrose agar was used as the conidiation medium. Microconidia were produced abundantly on this medium after seven days of incubation at room temperature. These were harvested and purified using methods described previously (Hashimoto et al., 1972). Microconidia were dislodged from the agar surface (7 to 10 days old culture) by scraping gently with a sterile policeman made of glass rod covered with a piece of tygon tubing. The conidia collected from 1 to 2 plates were then transferred to a sterile test tube (25 x 100 mm) containing 1 ml of sterile water and gradually were made into a

* This is a virulent strain isolated originally from a patient in Viet Nam and deposited in the American Type Culture Collection through our laboratory upon the request of ATCC following our publication (Hashimoto et al., 1972).

homogeneous suspension by gently rubbing the spores with the policeman against the test tube wall. Filtration of the conidial suspension through ten layers of cheesecloth proved useful in eliminating hyphae and clumped masses of conidia. Microconidia in the filtrate were washed 8 to 10 times until a microscopically clean conidial suspension was obtained.

Viability of conidia. Microconidia to be tested were inoculated into 2 ml of 1 % peptone solution and incubated at 37 C on a rotary shaker. Samples were withdrawn at intervals and examined by phase contrast microscopy for germination and vegetative outgrowth as described previously (Hashimoto et al., 1972).

Preparation of microconidial walls. Clean, purified, microconidia (ca. 5 ml heavy suspension) were mixed with 4.5 g of acid-washed glass beads (75-105 μ m, Sigma Chemical Co.) in a vial which was then shaken at 5 C in a Mickle cell disintegrator (H. Mickle Co.). Two to three 20 min full power treatments, with alternating 10 min resting periods were usually sufficient to achieve maximal breakage of conidia. To separate conidial walls from glass beads, the mixture was allowed to stand for 10 min at 4 C. The glass beads settled to the bottom and the supernatant containing broken microconidia was carefully pipetted into a clean tube. The conidial walls were separated from the cytoplasmic components by means of differential centrifugation and subsequently washed 10 to 12 times in distilled water. In order to eliminate cytoplasmic particles adhering to the walls, the washed conidial walls were subjected to mild sonication

for 30 sec (2 to 3 times) using a Sonifier (Branson Instruments Inc.). The wall fragments, recovered by centrifugation ($2,000 \times g$), were washed further with distilled water (5 times) until no extinction at 260 or 280 nm in the washings was detectable. All procedures were carried out in the cold (below 10 C) to minimize degradation of conidial walls by endogenous enzymes. The conidial walls prepared in this method are essentially free from unbroken conidia and other particulate materials as evidenced by phase contrast and electron microscopy. The contamination of the walls by membranous components of the cells is not completely eliminated but considered negligible for our purpose. The purified conidial walls were lyophilized and stored in a desiccator over P_2O_5 at room temperature. Materials used for electron microscopy studies were not dried.

Preparation of vegetative walls. 24 hr cultures of *T. mentagrophytes* grown in Sabouraud dextrose broth (4 % glucose, 1 % peptone) at 37 C on a rotary shaker were harvested by filtration on a $0.45 \mu m$ membrane filter (Millipore Corp.) washed several times with sterile distilled water, and resuspended in distilled water. The hyphae were disintegrated by sonication (Model S-75, Sonifier, Branson Instruments Inc.). Treatments for 1 min at full power (tap 6) were alternated with rest intervals of 4 min for cooling. The degree of disruption was checked microscopically. More than 15 treatments were necessary to obtain maximal breakage (99 %). The temperature of the suspension was maintained at approximately 4 C during the treatment by immersing the tube in a cold bath. The mycelial fragments were centrifuged at

2,000 x g for 10 min at 4 C and the pellet washed with water 15 to 20 times by means of differential centrifugation until microscopically clean walls were obtained. These were then freeze-dried and stored in a desiccator.

Dry weight determination. Wall suspension was filtered through pre-weighed, predried membrane (Millipore, 0.45 μ m pore size, Millipore Corp.), placed in aluminum planchets, and dried in an oven at 85 C to a constant weight.

Phase contrast microscopy. The morphology of untreated and chemically and enzymatically treated microconidial and hyphal walls were examined in a dark phase contrast microscope using an oil immersion objective (DM 100 x; numerical aperture, 1.25; Nikon). Photomicrographs were taken with a Nikon M-35S camera attached to the microscope using panchromatic film (plus-X, Eastman Kodak Co.).

Electron microscopy.

Ultrathin section. Walls were first fixed with glutaraldehyde (5 % in veronal acetate buffer, pH 6.1) for 6 to 10 hr at room temperature. After washing in the buffer three times, these were fixed at room temperature in 2 % osmium tetroxide (Kellenberger et al., 1959) overnight followed by uranyl acetate (0.5 %) for 1 hr. After dehydration through a graded acetone series, the walls were embedded in a mixture of Epon 812 and 815 and polymerized at 60 C for 22 hrs. Sections were cut with glass knives on an LKB 4800A ultratome (LKB Produkter AB) and mounted on formvar coated copper grids (300 mesh, Ernest F. Fullam, Inc.). After staining with lead citrate (Reynolds,

1963), sections were examined with a Hitachi HU-11A electron microscope operating at 50 or 75 KV.

Shadowed preparation. Wall samples were mounted on formvar coated copper grids and air dried. The grids were placed in a vacuum evaporator (Type HUS-3B, Hitachi Ltd.), shadowed with platinum-carbon and examined under the electron microscope.

Replica preparation. Samples were placed on formvar coated grids, shadowed with platinum-carbon first and then coated with evaporated carbon. After dissolving the formvar with chloroform, samples were floated off the grids on chromic acid (Dichrol, Scientific Products) (2 hr), cleaned with sodium hypochlorite (Matheson, Coleman and Bell Manufacturing Chemists.) (2 hr), washed thoroughly with distilled water, and transferred onto clean copper grids.

Freeze-etched preparation. These were made in a Balzers (Model BA 360 M, Balzer High Vacuum Corp.) by the method of Friedman et al., (1968) except that 4 to 6 % sodium hypochlorite was used in place of Eau de Javelle to clean the replica.

Renografin density gradient centrifugation of microconidial and hyphal walls. Renografin (Reno M-60, meglumine diatrizoate, E. R. Squibb and Sons, Inc.) linear gradients were prepared as previously described (Tamir and Gilvarg, 1966). Each 12 ml gradient (45 % to 60 % top to bottom) was layered over a 1 ml 60 % renografin cushion at the bottom of the centrifuge tube (15 ml, Pyrex brand, no. 8441). Samples of walls were layered on top of the gradient and centrifuged in a

Beckman centrifuge (Model J-21) with a JA-20 head at 18,000 rpm for 10 min (5 C). The individual bands formed in the gradient were withdrawn by a syringe and washed thoroughly with distilled water.

Criteria of purity of wall preparations. The purity of wall preparations is usually defined as the absence of cytoplasmic contamination. The methods used in our laboratory for such purposes were as follows:

Microscopic examinations. Wall preparations were examined by phase contrast microscopy. However, the absence of cytoplasmic components could be best detected by electron microscopy of the ultrathin sections prepared from the isolated walls.

Acridine orange stain. This dye differentially stains DNA and RNA (Socha, 1969). Under ultraviolet illumination, materials containing DNA-dye complex would appear bright yellow green while those with RNA-dye complex would appear crimson red. A stock solution of 0.1 % acridine orange (Allied Chemical and Dye Corp.) in McIlvaine's citrate-buffer (pH 4.0) was prepared and stored in a brown stopper bottle at 4 C. Immediately prior to use, a staining solution of 0.01% acridine orange in buffer was prepared from the stock. Impression smears were fixed in glacial acetic acid-methanol (3:1) for 7 min at room temperature, rinsed in buffer (pH 4.0), and stained with acridine orange for 7 min at room temperature. After staining, the coverslips were rinsed in two changes of buffer for 4 to 5 min and mounted directly on a clean glass slide with a fresh drop of buffer. The preparation was sealed with paraffin and examined with a Wetzlar

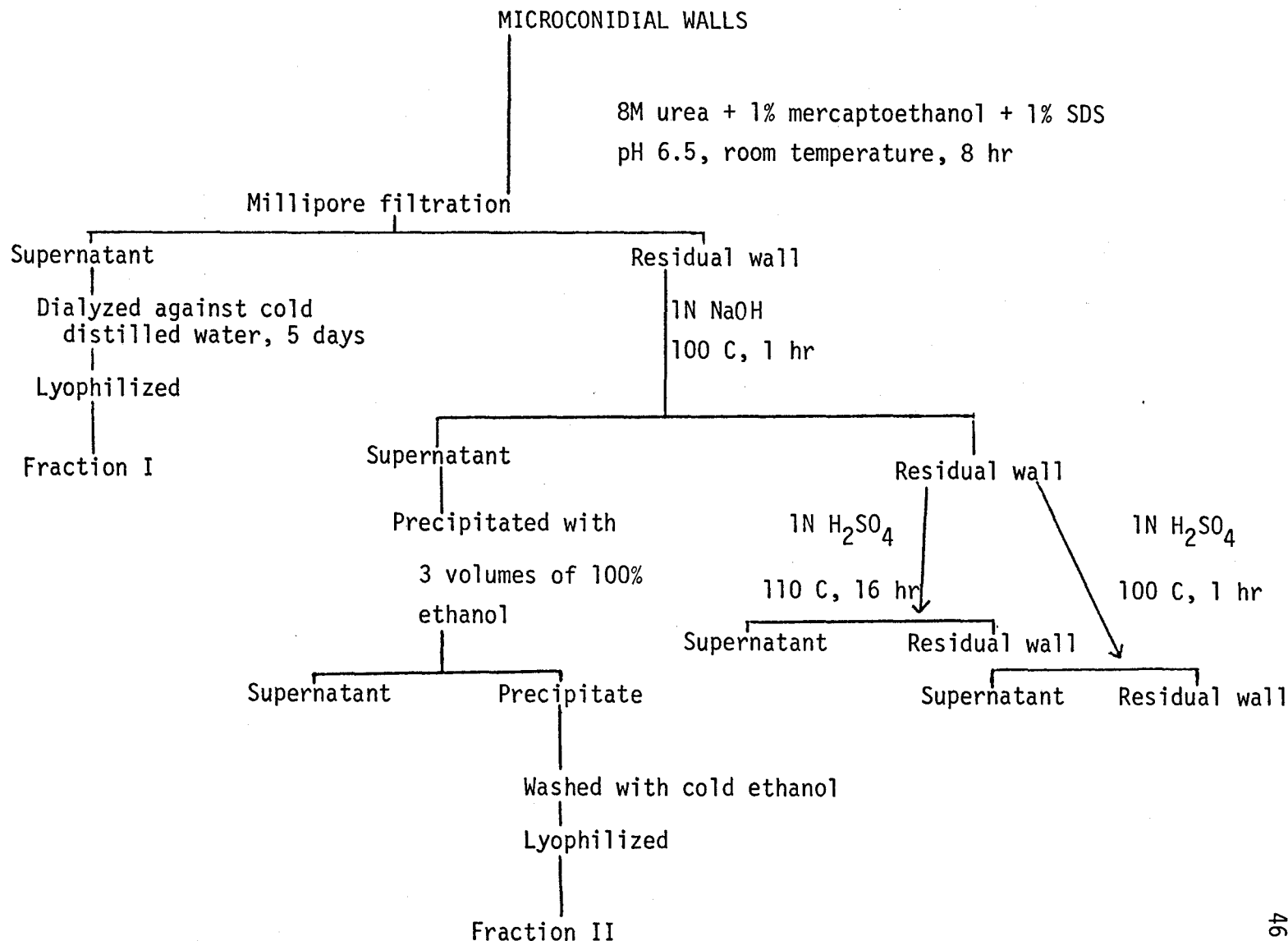
fluorescent microscope (Ernst Leitz Co.).

Ultraviolet absorbance. The absence of nucleic acids has usually been considered one of the most convincing pieces of evidence for purity of wall preparations. These were detected spectrophotometrically using a Gilford recording spectrophotometer (Model 2000, Gilford Instruments Laboratories). Wall preparations were considered free of aqueous extractable nucleic acids and proteins when no adsorption peaks at 260 nm and 280 nm were detected in the washings.

Chemical extraction and fractionation of conidial walls. For the isolation of wall polymers, the purified conidial walls were extracted sequentially with various chemicals as detailed in Fig. 1.

Urea, mercaptoethanol, and sodium dodecylsulfate (UMS) extraction. Microconidial walls were extracted with 0.1 M sodium phosphate buffer (pH 6.5) containing 8 M urea, 1 % mercaptoethanol, and 1 % sodium dodecylsulfate at room temperature with constant stirring for specified periods of time. The soluble fractions were separated from the residual walls by means of centrifugation (6,000 x g) followed by filtration through a millipore filter (0.45 μ m, Millipore Corp.). The extracted walls were washed thoroughly with distilled water and stored at 4 C until use. The supernatant was dialyzed against distilled water at 4 C for 5 days with frequent changes of water, lyophilized, and stored over P_2O_5 in a desiccator.

Fig. 1. Chemical extraction and fractionation of T. mentagrophytes microconidial walls.



Hot alkali extraction. Untreated walls or UMS extracted conidial walls (2 mg/ml) were boiled in freshly prepared 1 N NaOH in screw-capped tubes (Pyrex, 15 ml) with occasional agitation. The hot alkali-soluble wall polysaccharides were precipitated by adding 2 to 3 volumes of 100 % ethanol to the supernatant. The precipitate was washed three times with ethanol, lyophilized and stored over P_2O_5 in a desiccator. The alkali-insoluble wall residues were washed three times with distilled water, lyophilized and stored.

Determination of optimal hydrolysis conditions:

Sulfuric acid and hydrochloric acid have been most widely used for the hydrolysis of cell wall polysaccharides. Since the concentrations of acid used and duration of their treatment time at a high temperature differed from system to system, the elucidation of an optimal condition for the hydrolysis of I. mentagrophytes microconidial walls was systematically determined. The effects of these two acids on hexoses, amino sugars, and hexose-protein mixtures were also examined.

Effect of sulfuric acid or hydrochloric acid on glucose or glucosamine solution. A number of ampoules containing 1 mg of glucose (or glucosamine) and 2 ml of sulfuric acid or hydrochloric acid (1 N, 2 N, and 3 N) were heated at 110 C in an oven for 0, 4, 8, and 12 hr. At the end of each treatment, duplicate ampoules were withdrawn and 0.5 ml samples of hydrolysates from each ampoule were removed for quantitation of glucose or glucosamine concentrations. Controls containing 2 ml of water and 1 mg of glucose or glucosamine

were used for each treatment time.

Effect of sulfuric acid or hydrochloric acid on glucose in the presence of protein. Mixtures of glucose and albumin (1 mg each) were placed in a number of ampoules containing 2 ml of sulfuric or hydrochloric acid at various concentrations (1 N, 2 N, and 3 N). These were sealed and heated in the oven at 110 C for 0, 4, 8, and 12 hr. At the end of each treatment time, duplicate ampoules were withdrawn and the glucose concentration was determined.

Effect of temperature on various hexoses in the presence of 1 N sulfuric acid. Ampoules containing 1 mg each of glucose, galactose, mannose or glucosamine in 2 ml of sulfuric acid were heated in an oven at 110 C or 120 C. At the end of 12 or 16 hr, ampoules were withdrawn and the hexose concentrations remaining in each individual ampoule were quantitated by the anthrone method using each individual sugar as a standard.

Hydrolysis of microconidial walls. For the elucidation of an optimal condition for acid hydrolysis of microconidial wall, duplicate samples (1-2 mg) were heated at 110 C in 2 ml of 1 N sulfuric acid in sealed ampoules. At the end of 4, 8, 12, 16, and 24 hr, respective ampoules were withdrawn, insoluble residue removed, and the clear supernatant fluid assayed for total carbohydrate present in the supernatant by the anthrone method using glucose as a standard.

Other conditions tested for wall hydrolysis included: a) pre-digestion in concentrated sulfuric acid for 1 hr in the cold, diluted to 1 N, and heated at 110 C for 16 hr ("concentrated sulfuric acid-1

N sulfuric acid" method) and b) 1 N sulfuric acid at 110 C for 16 hr followed by concentrated sulfuric acid for 1 hr in the cold ("1 N sulfuric acid-concentrated sulfuric acid" method).

Paper chromatography. Acid hydrolysates of walls were neutralized with barium hydroxide and the precipitate removed by centrifugation. The supernatant fluid was concentrated in vacuo at room temperature and individual monosaccharides were determined by descending paper chromatography using Whatman no. 1 paper. The solvents used were n-butanol-acetic acid-water, ethanol-n-butanol-water, ethyl acetate-pyridine-water, and n-butanol-pyridine-0, 1 N HCl. The latter, at a ratio of 5:3:2 (by volume) gave consistent separation of monosaccharides in our system and was used throughout this study. Standard solutions of sugars were prepared in sodium benzoate saturated distilled water to prevent any microbial growth. Chromatograms were developed by reacting in a chromatography oven at 105 C after spraying with aniline oxalate (a mixture containing 9.3 g aniline/500 ml ethanol and 17.6 g oxalate /500 ml H₂O). For quantitative determination of individual monosaccharides, the method described by Chaykin (1966) was used. The non-revealed sugar spots (located on parallel chromatograms developed with aniline oxalate) were cut out, eluted with distilled water, and concentrations of the clarified eluates were determined by the anthrone method using the proper standard sugar solutions.

Thin layer chromatography. Chromatograms were run on silica gel sheets (Eastman Chromatogram sheet 6061, Eastman Kodak Co.), or on

precoated silica gel plates (Uniplate, Analtech Inc.) using solvent system containing n-butanol-pyridine-0.1 N HCl (5:3:2, by volume). Visualization of sugar spots was achieved with aniline oxalate spray.

Gas-liquid chromatography. Gas-liquid chromatography was employed using method described by Bolton et al. (1965). Microconidial walls were hydrolyzed with 1 N sulfuric acid at 110 C for 16 hr as described previously. After neutralization, the wall hydrolysate was lyophilized and treated with 10 to 100 μ l of silylation reagent (pyridine-hexamethyldisilazane-trimethylchlorosilane, 5:1:1 by volume) at room temperature for 10 min. Two to 4 μ l of the solution was injected into a Varian Aerograph model 1200 gas chromatograph. The glass column (3 mm by 1.8 m) was packed with 5 % SE-30-coated chromasorb W (60/80 mesh). Chromatography was carried out isothermally at 170 C with nitrogen as a carrier gas. Standards contained 0.75 to 2.25 μ g of trimethyl silylated derivatives prepared from authentic sugars (galactose, glucose, and mannose).

Spectrometry. Ultraviolet and visible spectra of various wall fractions were obtained using Gilford (model 2000, Gilford Instruments Laboratories Inc.), and Cary (Cary Instruments) spectrophotometers.

Disc gel electrophoresis. The method used for acrylamide gel electrophoresis of wall fractions was essentially the same as that described by Davis (1964). Glass columns (5 x 75 mm) were filled with 7.5 % lower separator gel (Acrylamide, Bio-Rad Laboratories) (55 mm in length) and upper 5.5 % spacer gel (7 mm). Samples

containing 100 to 200 μ g of material were suspended in 0.2 ml of spacer gel and layered on top of each tube. Electrophoresis were performed at 4 C in a Tris(hydroxymethyl)aminomethane(Tris)-glycine buffer (pH 7.5 and 8.3), or in Tris-citrate buffer (pH 6.6) at a constant current of 2 mA per tube. When the tracking dye had migrated 1 cm above the bottom of the gel column, electrophoresis was terminated. The gels were fixed in 12 % trichloroacetic acid (TCA) for 30 min, and stained with Coomassie blue (0.005 % in 12.5 % TCA). The excess dye was removed in 10 % TCA. The gels were also stained with periodic acid-Schiff reagent for carbohydrates (Fairbanks and Wallach, 1971), alcian blue for glycoprotein complexes, or sudan black B for lipid.

Gradient gel electrophoresis was carried out using commercially prepared gels (Gradipore, Isolab Gradient Division Inc.,) containing 2.5 to 27 % polyacrylamide with gradient cross-linking. Protein standards (kindly provided by Dr. C. F. Lange) used were hemoglobin (m.w. 60,000), human serum albumin (m.w. 67,000), and human IgG (m.w. 160,000). All gels were run at different pH values (7.5 and 8.3) with varying running periods (6 hr to overnight), and were terminated when hemoglobin standard migrated approximately to the end of the tube.

Enzyme digestion. Wall samples were incubated with digestive enzymes for desired period of time at room temperature. The digest was heated for 10 min in a boiling water bath to inactivate the enzyme, and centrifuged to remove the insoluble residue. The super-

natant was condensed to a minimal volume and components released by enzyme digestion were identified by paper chromatography described previously. The insoluble wall residues were hydrolyzed with 1 N sulfuric acid at 110 C for 16 hr and analyzed for individual monosaccharides present.

The enzymes used for this study included: chitinase(chitin glycanohydrolase, E.C.3.2.1.14, Sigma Chem. Co.); (1→3)-β-glucanase (β,1,3 glucan glucanohydrolase, E.C. 3.2.1.6, kindly provided by Dr. S. Nagasaki, Kochi University, Japan); and glusulase (a mixture of various hydrolytic enzymes, snail digestive enzyme, Endo Laboratories). Chitinase and glucanase were used without further purification. In order to eliminate the free sugars present in the enzyme preparation of glusulase, prefiltered glusulase was precipitated with 3 volumes of ice cold acetone. The mixture was filtered through sintered glass, washed thoroughly with ice cold acetone, dried in vacuo, and stored at -10 C. The purified acetone powder was rehydrated with sterile distilled water prior to use.

Test for proteolytic activity. The proteolytic activity of fraction-I was estimated using the method described by Tomarelli et al. (1949). Commercially obtained substrates: azoalbumin and azocasein (Sigma Chem. Co.) were used. The reaction mixture contained 0.2 ml of substrate solution (10 mg/ml), and 0.2 ml of appropriate amount of sample (fraction-I) or diluted pronase standard (12.5 to 50 μg). After incubation at 37 C for 30 min, the digestion was stopped and undigested substrate was removed from

solution by addition of 3 ml of TCA (5 %). After centrifugation, 2 ml of supernatant was added to 2 ml of 0.5 N NaOH and the color intensity was read at 440 nm on a spectrophotometer (Coleman, model 6/20, Coleman Instruments). A substrate blank was prepared by adding 0.2 ml of water to 0.2 ml of substrate solution and was treated in the same manner as the samples.

Chemical analyses.

Total carbohydrate. The anthrone method (Morris, 1948), was used for quantitation of various hexoses in the microconidial walls. Dried, preweighed wall samples (ranging from 1 to 2 mg) were placed in a 125 ml Erlenmeyer flask containing 25 ml of water and 50 ml of anthrone reagent (Sigma Chem. Co.) (0.2 % anthrone in concentrated sulfuric acid) were added (water: anthrone = 2:1). The mixture was boiled in a water bath for 10 min along with tubes containing glucose standard solutions. After rapid cooling in an ice bath, color intensities were measured in a Klett-Summerson photoelectric colorimeter (Klett manufacturing Co., Inc.) at 540 nm. When predigestion of walls with concentrated acid was necessary, walls were dissolved in 66 % sulfuric acid at room temperature for 10 to 30 min before analyzing for their total carbohydrate content.

Total glucosamine. A modification of the Elson-Morgan method (Winzler, 1955) was used for the glucosamine determination. Dried samples of walls were hydrolyzed in 2 ml of 6 N HCl in a screw-capped test tube in a boiling water bath. After 4 hr of hydrolysis, the mixture was centrifuged, and the supernatant was placed in a centri-

fuge tube (15 ml, Pyrex brand), neutralized with 6 N NaOH (to a final volume of 6 ml). To every 2 ml sample, 1 ml of freshly prepared acetylacetone reagent (1 ml of acetyl acetone in 50 ml of 0.5 N Na_2CO_3) was added and mixed. All tubes were capped with glass marbles and boiled in a water bath for 15 min. After cooling in an ice bath, 5 ml of 95 % ethanol were added and the contents mixed. One ml of Erlich's reagent (4 parts of 2.5 % para-dimethyl-aminobenzaldehyde in methyl cellosolve and one part of concentrated HCl) was then added to each sample tube followed by 1 ml of 95 % ethanol. Readings were taken after 30 min at 530 nm using a spectrophotometer (Spectronic 70, Bausch and Lomb Co.).

Chitin. Chitin was quantitatively determined by the method described by Blumenthal and Roseman (1957). Dried walls were placed in heavy-walled, screw-capped conical tubes (12 ml) and subjected to sequential extractions with 2 ml each of the following: 10 % NaOH at 100 C for 30 min; water; 2 % HCl at room temperature for 1 hr; 10 % NaOH at 100 C for 30 min; and water. Each extraction was followed by centrifugation and removal of the supernatant fluid. The washed residues were then hydrolyzed in 2 ml of 6 N HCl (4 hr at 100 C) and analyzed for total glucosamine content as described previously.

Amino acid. Samples of wall (1.5 to 2.5 mg) were weighed into test tube (Pyrex) and 2 ml of 6 N HCl was added. After freezing the suspension in a dry ice-acetone mixture, the ampoules were evacuated using a water aspirator and sealed. These were then hydrolyzed for 22 hr at 110 C. After removal of the acid and water in vacuo over

NaOH pellets, the dried hydrolysate was dissolved in pH 2.2 buffer and analyzed for amino acids using a Spinco Model 120 C Amino Acid Analyzer (Speckman et al., 1958).

Protein. Total protein was estimated by the method of Lowry et al. (1951) using crystalline bovine serum albumin (Sigma Chem. Co.) as a standard. Phenol reagent (Folin Ciocalteu) was purchased from Anderson Laboratories, Inc. Actual protein concentrations were calculated from recoveries on amino acid analysis.

Lipid. The total lipid content of wall samples were determined by the method of Folch et al. (1957). Preweighed, dried conidial walls were extracted with chloroform-methanol mixture (2:1) for 16 hr at room temperature. The extract was separated into two phases by adding 0.37 % KCl (at a final concentration of 20 % v/v). The upper phase was withdrawn and the lower phase washed three times with chloroform:methanol:water (3:47:48, by volume). The lower phase was evaporated to constant weight representing the total amount of extracted lipids.

Phosphorus. Total phosphorus content was estimated by the ashing procedure described by Ames (1966). To 0.01 to 0.1 ml of sample in a test tube (Pyrex, 13 x 100 mm), 0.03 ml of magnesium nitrate solution [$10\% \text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in 95 % ethanol] was added. The material was taken to dryness and ashed by shaking the tubes over a strong flame until the brown fumes disappeared. After adding 0.3 ml of HCl (0.5 N), the tubes were capped with glass marbles and heated in a boiling water bath for 15 min. After the

tubes were cooled, 0.7 ml of Mix solution (10 % ascorbic acid in water: 0.42 % ammonium molybdate in 1 N H_2SO_4 = 1:6) was added and the tubes incubated 20 min at 45 C, or 1 hr at 37 C. Readings were taken at 820 nm. A phosphorus standard solution containing 20 μg of phosphorus (as KH_2PO_4) per ml (Sigma Chem. Co.) was used.

Ash. For ash determination, walls were placed in predried, accurately weighed platinum crucibles and heated at 600 C in an oven till constant weights were obtained.

CHAPTER III

RESULTS

A. Determination of optimal condition for acid hydrolysis.

1. Determination of acid used and condition for hydrolysis.

Effect of sulfuric or hydrochloric acid on glucose. As shown in Fig. 2a, no significant destruction of glucose was observed after 4, 8 and 12 hr of heating in the presence of 1 N or 2 N sulfuric acid. A 25 % decrease in concentration occurred after prolonged period of treatment (12 hr) with a higher concentration of sulfuric acid (3 N). In contrast to this, a marked destruction of glucose was seen when hydrochloric acid was used under the identical experimental conditions (Fig. 2b). More than 25 % loss of glucose was observed after only 4 hr of hydrolysis in 1 N HCl, at 110 C, and as the concentration of the acid and duration of treatment increased, the loss of the sugar was even more apparent. Less than 10 % and 20 % of the original concentration of glucose was left after 12 hr at 110 C in 3 N and 2 N HCl respectively.

Effect of sulfuric or hydrochloric acid on amino sugar. Since amino sugar polymers such as chitin are often reported in fungal walls, the effects of sulfuric or hydrochloric acid (1 N, 2 N, and 3 N) and treatment times (at 110 C) on glucosamine were studied. Although data are not shown here, neither acid at any concentration tested showed any degradative effect on glucosamine with heating time up to 12 to 16 hr.

Fig. 2a. The effect of concentration of sulfuric acid on the destruction of glucose (at 110 C). A number of ampoules containing 1 mg of glucose and 2 ml of sulfuric acid were heated at 110 C for 0, 4, 8, and 12 hr. At the end of each treatment, duplicate ampoules were withdrawn and 0.5 ml of samples of hydrolysates were removed for quantitation of glucose using the anthrone method. Controls containing 2 ml of water and 1 mg of glucose was used for each treatment time. Symbols: Δ , H_2O ; x, 1 N sulfuric acid; o, 2 N sulfuric acid; and \square , 3 N sulfuric acid.

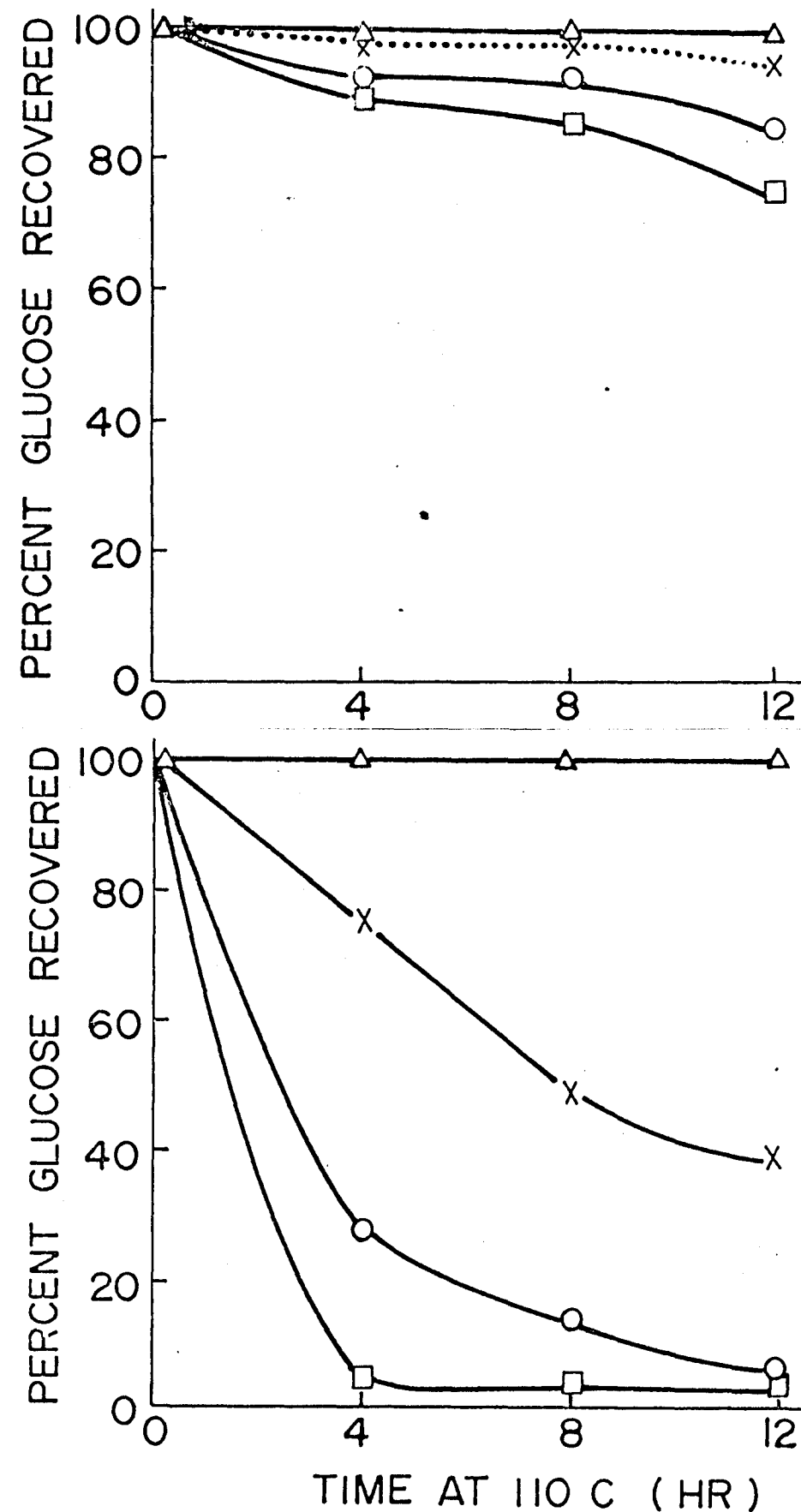


Fig. 2b. The effect of concentration of hydrochloric acid on the destruction of glucose (at 110 C). Experimental conditions were similar to that described in Fig. 2a, except hydrochloric acid was used. Symbols: Δ , H_2O ; x, 1 N HCl ; o, 2 N HCl ; and \square , 3 N HCl .

Effect of sulfuric or hydrochloric acid on glucose in the presence of protein. Since the susceptibility of sugars to acid degradation might differ if they are complexed with protein or proteins that are present in a hydrolytic mixture, the effect of hot acid treatment (sulfuric or hydrochloric acid at 110 C) on glucose in the presence of protein (albumin) was examined. Although both hydrochloric acid and sulfuric acid caused degradation of glucose, they exerted less destructive effect on monosaccharides when protein was present (Fig. 3).

Effect of 1 N sulfuric acid on glucose, mannose, and galactose. Results obtained above showed that sulfuric acid was less destructive to glucose than was hydrochloric acid. Therefore, 1 N sulfuric acid was preferentially used in subsequent hydrolysis experiments. Since a preliminary experiment revealed that glucose, galactose and mannose were the only neutral sugars present in the wall hydrolysate of I. mentagrophytes, the effect of 1 N sulfuric acid on these sugars were investigated in a similar manner. As shown in Table 2, treatment by 1 N sulfuric acid at 110 C for up to 16 hr did not cause significant degradation of any of the three hexoses tested, although mannose seemed to be more readily degraded (ca. 15 % loss). Noticeable destruction of all hexoses occurred when the temperature was raised to 120 C.

Effect of acid hydrolysis on chromatographic mobilities (R_f value of monosaccharides). It has been reported that degradation or interconversion of sugars can result from treatment in acid at high

Fig. 3. The effects of sulfuric acid or hydrochloric acid on glucose in the presence of protein (at 110 C). Mixtures of glucose and albumin (1 mg each) were placed in ampoules containing 2 ml of sulfuric or hydrochloric acid at various concentrations (1 N, 2 N, and 3 N). After heating at 110 C for 4, 8, and 12 hr, the glucose remaining in each ampoule was determined by the anthrone method. Symbols: x; H_2O control; ●, 1 N H_2SO_4 ; ▲, 2 N H_2SO_4 ; ■, 3 N H_2SO_4 ; o, 1 N HCl ; Δ, 2 N HCl ; □, 3 N HCl .

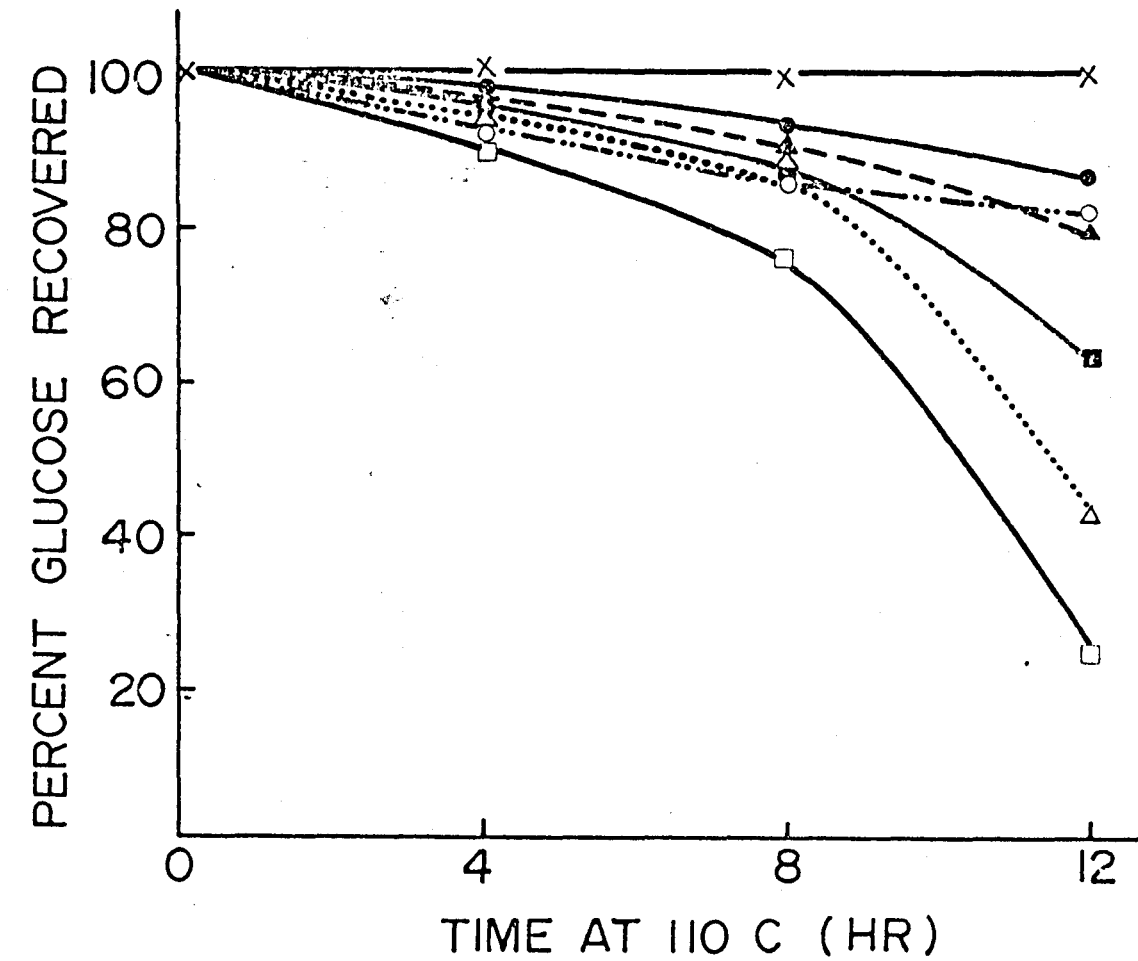


TABLE 2. The effect of temperature and heating time on glucose, galactose, and mannose in the presence of 1 N sulfuric acid^a.

	Percent sugar recovered ^b			
	Hydrolysis at			
	110 C		120 C	
	Hydrolysis time			
	0 hr	12 hr	16 hr	16 hr
Glucose	100	97.0	96.4	20.0
Galactose	100	98.0	91.4	17.0
Mannose	100	98.0	85.2	18.8

^a Ampoules containing 1 mg each of glucose, galactose, and mannose in 2 ml of sulfuric acid (1 N) were heated in an oven at 110 C or 120 C. At the end of 12, and 16 hr, ampoules were withdrawn and the hexose concentrations remaining in each individual ampoule were quantitated by the anthrone method using each individual sugar as a standard.

^b Values represent the percent sugar recovered after each individual treatment.

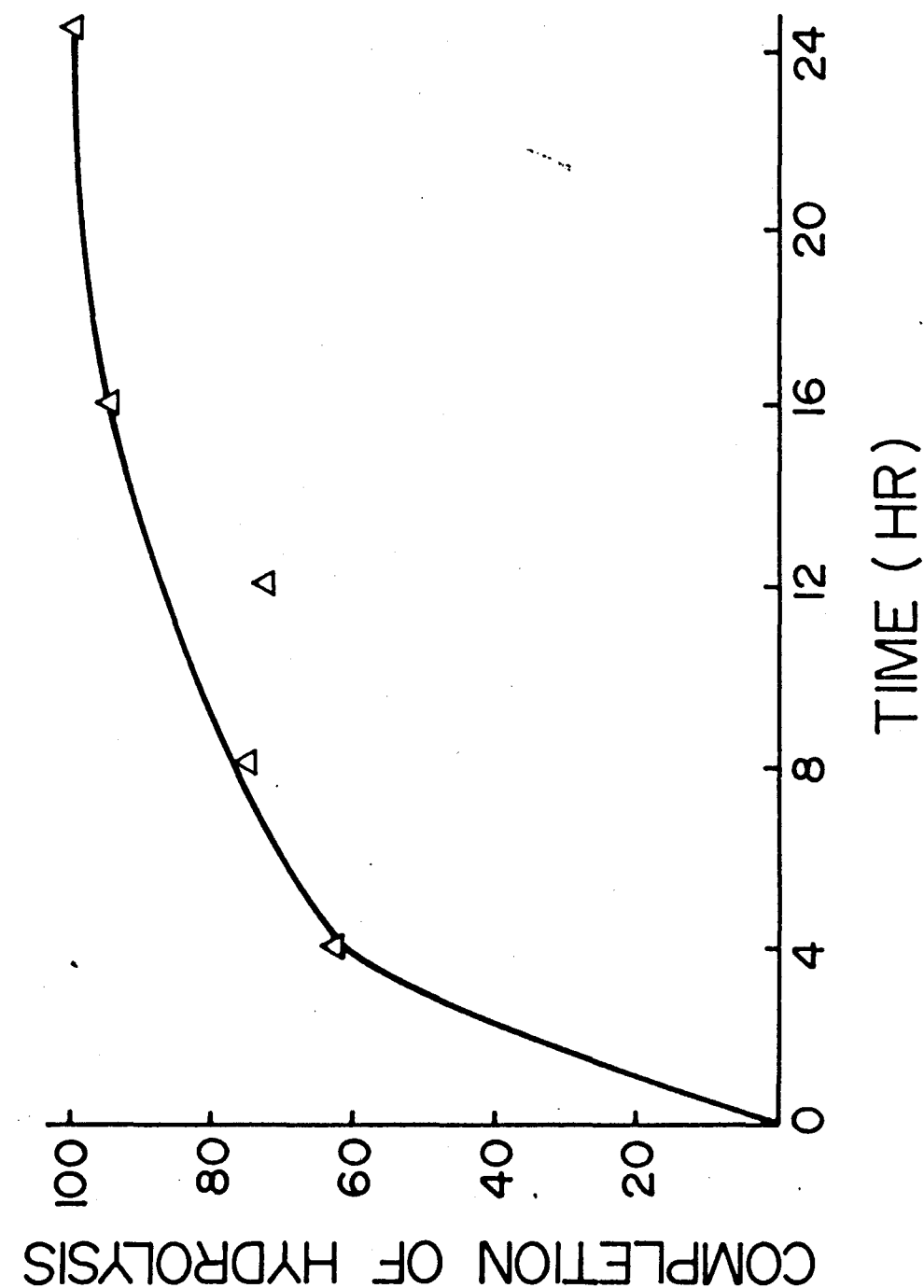
temperature. However, under the experimental conditions employed, glucose, galactose, mannose or glucosamine were not degraded after heating at 110 C in sulfuric acid for 16 hr. R_f values of these sugars on paper chromatogram remained identical to the values of the corresponding untreated standards.

On the basis of these preliminary experiments, 1 N sulfuric acid was chosen as the hydrolytic agent for all subsequent experiments involving hydrolysis.

2. Determination of an optimal condition for the hydrolysis of microconidial walls of I. mentagrophytes.

Microconidial walls of I. mentagrophytes were hydrolyzed with 1 N sulfuric acid at 110 C for 4, 8, 12, 16, 20 and 24 hr. As illustrated in Fig. 4, anthrone positive materials were released from the walls progressively as the time of hydrolysis increased reaching a plateau at 16 hr followed by a slight additional increase (1 %) after prolonged treatment (24 hr). Approximately 80 % of wall carbohydrate was released, as detected by the anthrone method using glucose as a standard. Other conditions tested for wall hydrolysis included the "concentrated sulfuric acid-1 N sulfuric acid" and the "1 N sulfuric acid-concentrated sulfuric acid" methods. When the former condition was used, I. mentagrophytes microconidial walls were first subjected to digestion with concentrated sulfuric acid (1 hr in the cold) before hydrolysing with 1 N sulfuric acid at 110 C for 16 hr. An increase of 4 % in carbohydrate release (as glucose) was observed. However, when similar hydrolysis condition was performed

Fig. 4. Percentage of carbohydrate release (as glucose) during acid hydrolysis (1 N sulfuric acid, 110 C) of microconidial walls of *T. mentagrophytes*. Ampoules containing microconidial walls and 1 N sulfuric acid (1 mg/ 2 ml) were heated at 110 C for 4, 8, 12, 16, and 24 hr. At the end of respective treatment time, duplicate ampoules were withdrawn, insoluble residue removed, and the clear supernatant assayed for total carbohydrate present by the anthrone method using glucose as a standard. Percent of carbohydrate release was expressed as the percent release relative to that amount release after 24 hr.



on individual sugar standards (glucose, galactose, and mannose), a destructive effect was noted. Although not shown here, a 15 to 20 % loss in sugar recoveries took place. Therefore, this method was not employed. The "1 N sulfuric acid-concentrated sulfuric acid" method was not feasible due to the technical error introduced, since it involved the manipulation of minute quantities of acid hydrolysed residue (after 16 hr hydrolysis in 1 N sulfuric acid at 110 C). Therefore, hydrolysis in 1 N sulfuric acid for 16 hr at 110 C appeared to be optimal for conidial walls of T. mentagrophytes, and was used as the standard method for all subsequent experiments.

B. Physical, morphological, and chemical characterization of microconidial walls of T. mentagrophytes.

1. Purity and some physical characterization of isolated conidial walls of T. mentagrophytes.

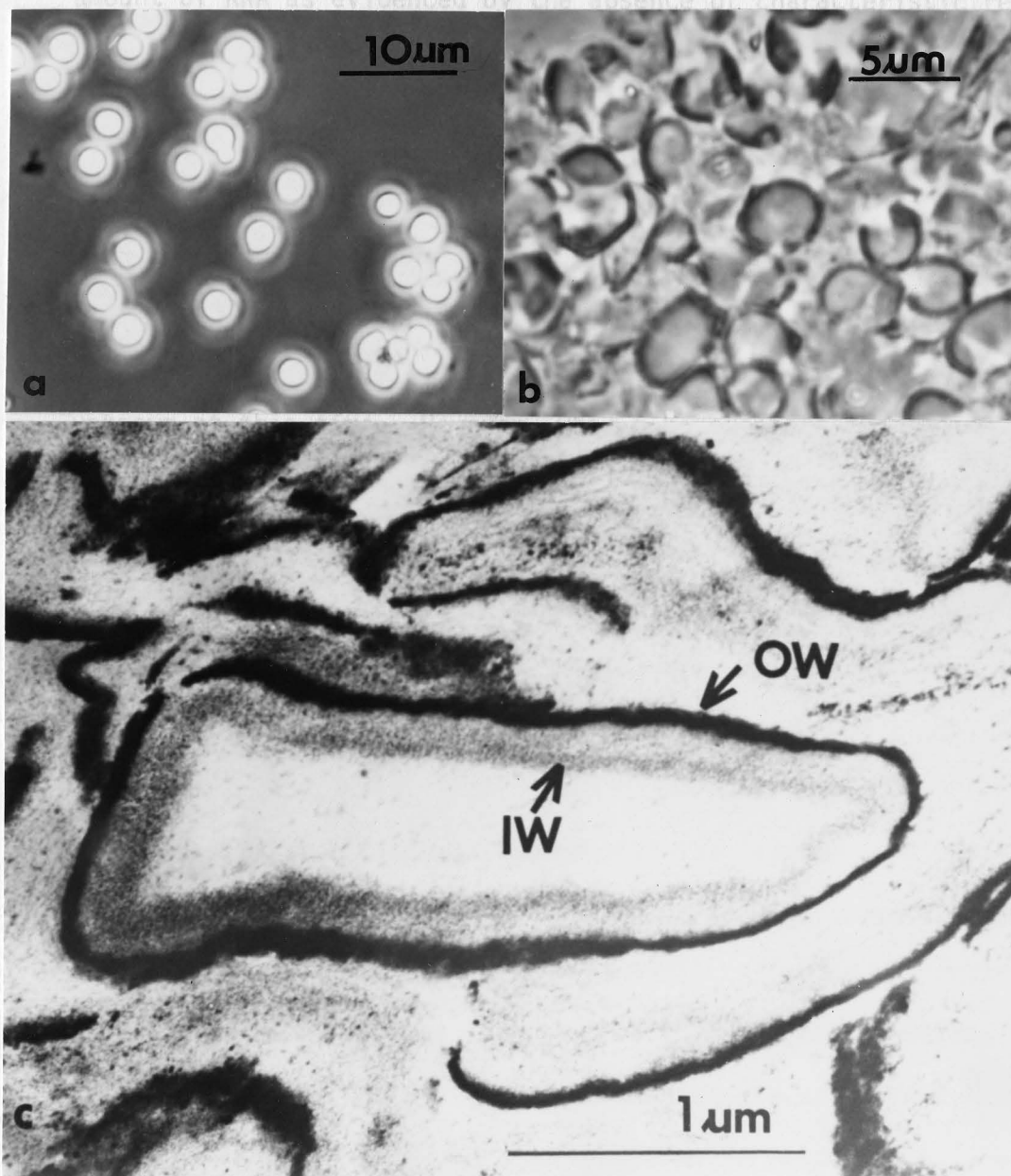
The purified conidia served as excellent starting materials for the preparation of wall fractions (Fig. 5a).

The use of a Mickle cell disintegrator produced total microconidial rupture and was satisfactory in fragmenting walls into several pieces. (Fig. 5b). Though occasional conidia or cytoplasmic particles were detected, the amount of such contamination can be safely estimated to be far below 1 % and regarded as negligible. Ultrathin sections of the isolated microconidial walls (Fig. 5c) showed that the preparation was essentially free from cytoplasmic contaminations, and that the walls still retained their structural characteristics. Other methods used, beside the micro-

Fig. 5a-b. Dark phase contrast photomicrographs of the T. mentagrophytes microconidia and their walls. a, isolated and purified microconidia; and b, isolated microconidial walls.

Fig. 5c. Electron micrograph of ultrathin section of the purified microconidial wall preparation. Note that the walls retained their original structural characteristics and the preparation was essentially free from cytoplasmic contamination. OW, outer wall; and IW, inner wall.

scopic examination, confirmed the parity of the walls. When stained with acridine orange, the conidial walls contained no detectable amount of RNA as evidenced by the absence of characteristic red



position than that of the conidial walls.

scopic examination, confirmed the purity of the walls. When stained with acridine orange, the conidial walls contained no detectable amount of RNA as evidenced by the absence of characteristic red fluorescence when examined under the fluorescence microscope.

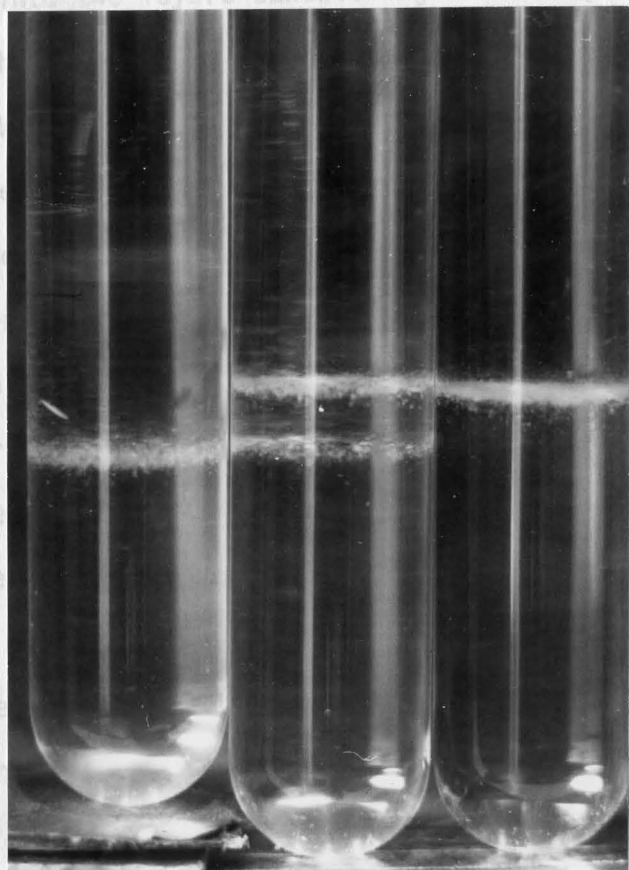
Lyophilized wall preparations were whitish yellow to brown in color and fluffy in appearance. They were somewhat hydrophobic. When suspended in water or buffer, unlike that of the dormant intact microconidia, clumping was often observed. A similar phenomenon has been observed in germinated conidia: the reason for which is still unknown at this time. However, when a homogeneous suspension of the walls was needed for chemical extraction or fractionation procedures, it could be obtained by a brief sonication or ultrasonic treatment (30 sec) of the clumped wall suspension. When stained with PAS (periodate acid-Schiff reagent), the conidial walls showed the strongly positive reaction indicating the presence of a considerable amount of carbohydrate.

When the purified wall suspension was layered on a renografin density gradient, only one band was observed upon centrifugation. Although the exact density of the conidial walls was not determined, they seemed to be lighter than those of the vegetative walls. As shown in Fig. 6, when a mixture of conidial and hyphal walls were layered on the gradient, the vegetative walls banded at a lower position than that of the conidial walls.

Fig. 6. Separation of microconidial walls and hyphal walls of T. mentagrophytes by linear renografin density gradient centrifugation. From left to right: tube containing only hyphal walls, tube containing a mixture of microconidial and hyphal walls, and a tube containing the microconidial walls. The gradient range of renografin was 45 % at the top and 60 % at the bottom.

2. Ultrastructure of the microconidial walls. 69

Electron microscopy of an ultrathin section through a microconidium revealed that the conidial wall consisted of at least two distinct layers (Fig. 7). The outer wall (OW), being electron dense and measuring 25 to 50 nm, can be further divided into two layers sandwiching a less electron dense middle.



The inner wall (IW), which measured 250 to 300 nm, was less electron dense. However, under ultrathin sectioning (Fig. 5c), under ultrathin sectioning and lost its structure. The outer surface of the inner surface was covered with fine fibrils (Fig. 8). The microconidia of *Aspergillus* microconidia showed short fibers and other structures. It has been observed on various other fungal spore surface, this is

2. Ultrastructure of the microconidial walls.

Electron microscopy of an ultrathin section through a dormant microconidium revealed that the conidial wall consisted of at least two distinct layers (Fig. 7). The outer wall (OW), appearing electron dense and measuring 25 to 50 nm, can be further resolved into two layers sandwiching a less electron dense middle layer. These two outer electron dense layers were designated as OW1 and OW2. In contrast to this, the inner wall (IW), which comprised the bulk portion of the conidial wall, measured 250 to 300 nm in thickness. It was of relatively low electron density. However, this inner wall was stainable with heavy metal (Fig. 5c), under which condition, the outer wall was stained too heavily and lost its characteristic two-layered appearance.

When shadowed with carbon-platinum, the outer surface of the wall appeared rough and amorphous, whereas the inner surface was sparsely covered with randomly oriented microfibrils (Fig. 8).

The freeze-etched surface of I. mentagrophytes microconidia showed the presence of rodlet-patches made up of short fibers oriented in juxtaposition (Fig. 9). Although similar structures have been observed on various other fungal spore surface, this is the first demonstration of such structures on the dermatophyte spores.

3. Chemical composition of microconidial walls.

Chemically, the microconidial walls contained 80 to 90 percent polysaccharides with lesser amounts of protein and lipid.

Fig. 7. Electron micrograph of an ultrathin section of a portion of a dormant microconidium showing the multiple-layered structure of the microconidial wall. The outer wall can be further resolved into two distinct electron dense layers (OW1 and OW2) sandwiching a middle electron less dense layer. The inner wall (IW) is of light electron density and is thicker. M, mitochondrium; L, lipid body.

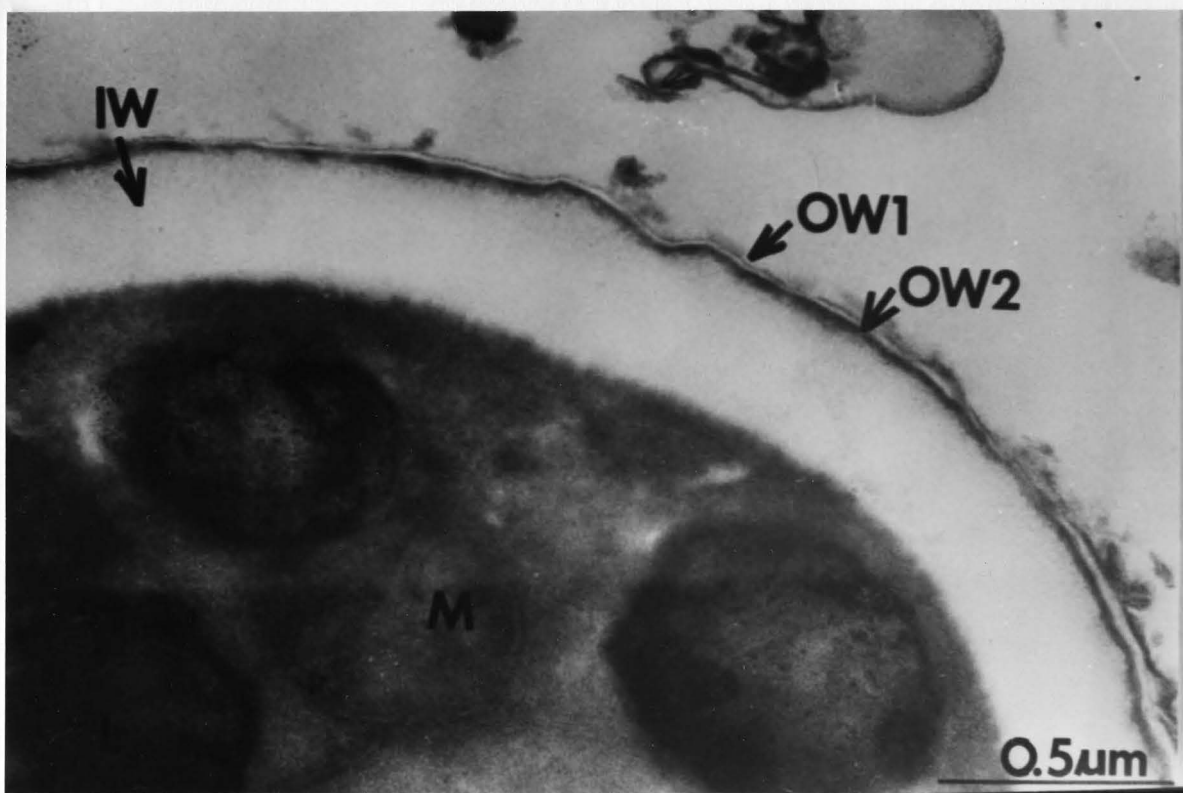
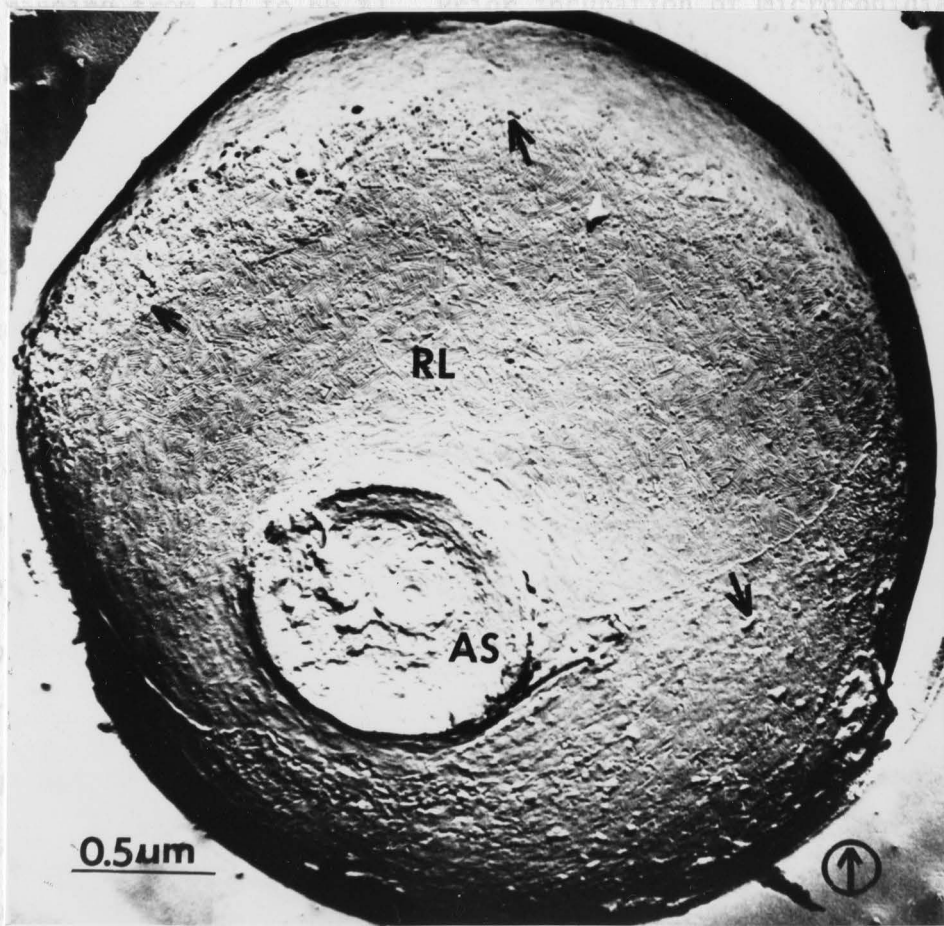
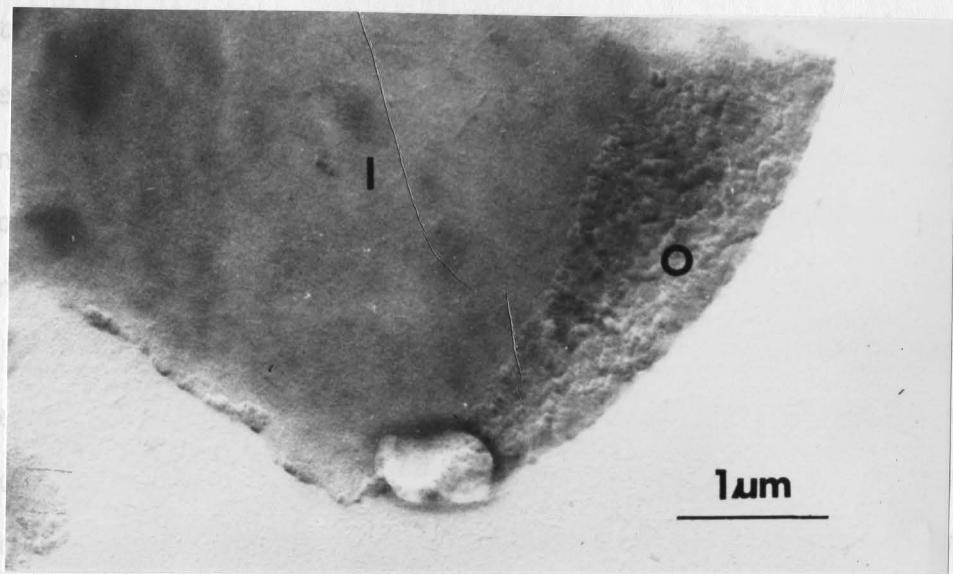


Fig. 8. Electron micrograph of the carbon-platinum shadowed wall fragments of T. mentagrophytes microconidia. The outer surface (O) is rough and amorphous while the inner surface (I) is sparsely covered by microfibrils.

Fig. 9. Electron micrograph of a freeze-etched replica of the surface of a dormant microconidium of T. mentagrophytes. The surface is covered by a layer of tightly woven rodlets oriented at juxtaposition. The presence of an outer filmy layer (arrows) can also be seen. RL, rodlet layer; and AS, abscission scar.



Other minor components such as phosphorus, pigment, and inorganic ash were also present (Table 3). A more detailed description of each individual component of the microconidial walls will be given below. For the purpose of comparison, those of the vegetative walls are also analyzed and presented in this section.

Carbohydrate. The anthrone method, using glucose as a standard, served to assess the total amount of hexosan present in the microconidial walls. Results obtained from repeated direct anthrone measurements indicated an average value of 63.4 % wall dry weight (ranging from 60 to 66 %). Prior incubation of microconidial walls in sulfuric acid (66 %) at room temperature for 10 to 30 min before the addition of anthrone did not increase the total glucose detected. Hyphal walls had an average content of 71 % of neutral sugars expressed as glucose equivalents higher than that of the microconidial walls (Table 3).

Neutral sugars. Monosaccharide constituents of wall hydrolysates were determined by paper chromatography. Among the five solvent systems tested for paper chromatographic studies, n-butanol: pyridine: 0.1 N HCl (5:3:2, by volume) proved to be the best and provided reproducibly excellent separations of individual sugars. The microconidial wall contained three neutral sugars: glucose, galactose, and mannose, with glucose being the major constituent sugar. The visual estimation of color intensities of sugar spots on paper chromatogram was used to estimate the relative ratio of monosaccharides present in wall hydrolysate. According to this

Table 3. Chemical composition of microconidial and hyphal walls of T. mentagrophytes.^a

Wall Component	% Dry weight ^b	
	Microconidial wall	Hyphal wall
Total carbohydrate	79.4	88.2
Neutral sugars ^c	63.4	71.1
Chitin	16.0	17.1
Protein	22.6	9.7
Lipid	3.5	3.9
Ash	1.7	5.6
Phosphorus	0.2	0.1

^a Methods for individual analysis were described in the Materials and Methods section.

^b Numbers represent percent dry weight of intact wall.

^c Values expressed as glucose equivalent by the anthrone method.

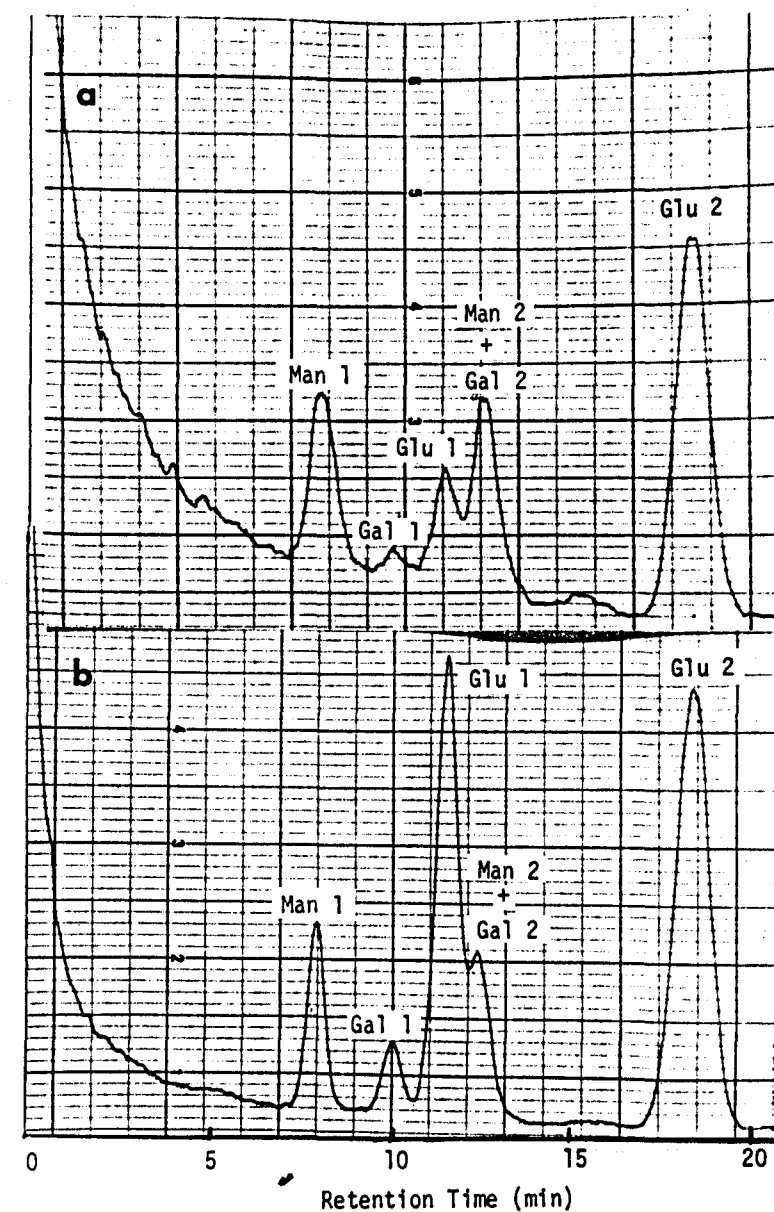
method, the ratio of galactose, glucose, and mannose was trace:3:1.

Although galactose was consistently observed on paper chromatograms of wall hydrolysates, the spot was always faint, and at times, hard to visualize. To further confirm the presence of galactose, gas-liquid chromatography was used. A reference chromatogram of a mixture of the three sugars is shown in Fig. 10a. Each of the three standard sugars were well separated on the column. Gas-liquid chromatographic analysis of the trimethylsilylated derivatives of wall hydrolysate of I. mentagrophytes microconidia showed the presence of five peaks. (Fig. 10b). Each peak had retention times which corresponded to authentic samples of the trimethylsilylated derivatives of mannose, galactose, and glucose. Similarly to results obtained from paper chromatography studies, glucose was the major fraction present in the hydrolysate of microconidial walls. The presence of galactose was trace but apparent. For further confirmation of peak 2 and 4 being galactose, the wall hydrolysate was co-chromatographed with an internal galactose standard. The enhancement of peaks 2 and 4 were noted (data not shown). Although not shown here, the elution profile of vegetative wall hydrolysate obtained from GLC was similar to that of the microconidial wall.

Amino sugar. Hexosamine constitutes about 18 % of the microconidial wall as determined by the Elson-Morgan method. The hexosamine was paper chromatographically identified as glucosamine only. No other amino sugars such as mannosamine or galactosamine were detected on the paper chromatograms. During analysis of amino acid

Fig. 10a. Gas-liquid chromatographic (GLC) separation of trimethylsilylated derivatives prepared from sugar standard containing mixture of mannose, galactose, and glucose. Gas-liquid chromatography was performed on glass column packed with 5 % SE-30-coated chromasorb W at 170 C constant temperature using a flame ionization detector system in a Varian Aerograph model 1200 gas chromatograph. Retention times (min) of individual sugar standards were as follows: mannose 1 (α -mannose) and 2 (β -mannose), 7.9 and 12.1; galactose 1 (α -galactose) and 2 (β -galactose), 10 and 12.5; and glucose 1 (α -glucose) and 2 (β -glucose), 11.3 and 17.7.

Fig. 10b. Gas-liquid chromatography elution profile of trimethylsilylated derivatives of acid hydrolysate of I. mentagrophytes microconidial walls.



content of conidial wall, glucosamine peak was the only amino sugar peak detected by the analyzer. It seemed, therefore, that glucosamine represented the only amino sugar present in the walls and this is in agreement with data obtained from the paper chromatography study. Similar results were observed for the vegetative walls.

Chitin (N-acetylglucosamine polymer). For more accurate estimation of the chitin content, the microconidial walls were subjected to alternating alkali and acid extractions (described previously using the method of Blumenthal and Roseman, 1957) prior to acid hydrolysis by 6 N HCl. The amount of glucosamine left in the residual wall after such extractions was approximately 16 % of the intact walls suggesting the presence of a considerable amount of a fully acetylated, insoluble glucosamine polymer (chitin). Since glucosamine could not be detected in any fractions other than fractions containing chitin, it is reasonable to suggest that the total amino sugar present in the conidial walls exists in the form of chitin. A similar chitin content was observed in the vegetative walls (Table 3). The presence of chitin in the microconidial walls was further confirmed by an enzymatic method; release of N-acetyl glucosamine was observed when microconidial walls were incubated in the presence of chitinase.

Amino acids. When the Lowry method was used for the quantitation of total protein, relatively low values were obtained, due to the possible insufficient solubilization of proteins from the wall material. When complete amino acid analyses were carried out on

wall hydrolysates (6 N HCl at 110 C for 22 hr) using ion-exchange column chromatography, a significant increase in total amino acid recoveries was observed. The total amino acid content of conidial wall was 22.6 %. As shown in Table 4, data revealed the presence of normal protein amino acids. When compared with those of the vegetative walls, the most significant difference appeared to be the total amino acid content of the two types of walls. Conidial walls contained twice the quantity of total amino acids as hyphal walls (9.7 %). In both types of walls, aspartic acid and glutamic acid accounted for more than 24 % of the total recovery (27.5 % in conidial wall and 23.7 % in the hyphal wall), sulfur-containing amino acids (cysteine, cystine, and methionine) were relatively low in quantities, and the presence of histidine was almost nil. Interestingly, lower amounts of arginine, tyrosine and proline were found in the microconidial walls.

Lipid. The amount of lipid extractable by chloroform and methanol (2:1) from microconidial wall was 3.5 % (percent dry weight of wall). Similar values were observed for those of the vegetative wall (3.9 %).

Phosphorus. Inorganic phosphorus appeared to be a very minor component of the conidial wall; it amounted to 0.17 % of the wall dry weight. An even lesser amount was detected in the vegetative walls (0.07 %).

Ash. The ash content estimated in the conidial walls was 5.6 %. Its nature and composition is not known at this time. A

TABLE 4. Amino acid composition of T. mentagrophytes microconidial and hyphal walls.

Amino acid	% μ moles	
	Microconidial wall	Hyphal wall
Alanine	8.1	8.8
Arginine	2.3	4.5
Aspartic acid	15.5	12.6
Cysteic acid	0.1	trace
Glutamic acid	12.0	11.1
Glycine	7.8	8.3
Half cystine	3.8	0.1
Histidine	trace	trace
Isoleucine	4.0	4.7
Leucine	7.7	7.9
Lysine	5.5	5.2
Methionine	0.8	1.3
Phenylalanine	3.3	3.5
Proline	4.8	6.4
Serine	7.5	8.4
Threonine	6.8	7.3
Tyrosine	1.9	3.0
Valine	7.3	6.2

lower value was found in the vegetative wall (1.9 %).

Pigment. As described previously, the conidial walls appeared yellowish or light brownish in color. The pigment was found to be extractable with hot alkali, but not with acids; its properties were not elucidated at this stage.

C. Chemical extraction and fractionation of microconidial walls of I. mentagrophytes.

The fractionation procedure used in our laboratory (Fig. 1) permitted separation of the microconidial wall into four principal fractions, the relative distribution of which is shown in Fig. 11. All fractions were then characterized chemically by qualitative and quantitative analyses.

1. Urea (8 M)-mercaptoethanol (1 %)-SDS (1 %) extraction. The presence of an urea-mercaptoethanol-SDS soluble protein or class of proteins possibly located in the spore coat, was reported to be a component of bacterial spores (Sommerville et al., 1970). In spores of Bacillus thuringiensis, this protein was shown to exist in a crystal-like structure (Delafield et al., 1968). Considering the similarity in appearance of the rodlets observed on freeze-etched surface of I. mentagrophytes microconidia (Fig. 9) and the bacterial spore coat protein, it was of interest to investigate whether the former could be solubilized using the same extraction technique. This was the reason for choosing UMS treatment as the first step of fractionation procedure for the microconidial walls (Fig. 1).

The optimal condition for this extraction under which maximal release of wall material occurred was first determined. Microconidial walls were extracted at room temperature with 0.1 M sodium phosphate buffer (pH 6.5) containing urea (8 M), mercaptoethanol (1 %), and SDS (1 %) for 4, 8, 12, and 18 hr. At the end of 8 hr extraction, release of UMS soluble material from the conidial walls reached a plateau under this condition. This extraction removed approximately 28 to 30 % of the intact wall dry weight. Essentially no material was extracted from walls by water or phosphate buffer (0.1 M, pH 6.5) alone. In the subsequent discussion, the extraction of microconidial walls using 8 M urea, 1 % mercaptoethanol and 1 % SDS at room temperature for 8 hr (pH 6.5) will be referred to as the "UMS extraction".

Fraction I. As described above, this fraction accounted for 28 to 30 % of the initial wall dry weight. The fraction, when lyophilized, was off-white in color. It was only partially soluble in water or in weak acid, but completely soluble in alkali.

Chemical analysis of this fraction showed the presence of 20 % anthrone positive carbohydrate (as glucose), 34 % protein (as estimated with the Lowry method), and 21 % lipid.

Disc gel electrophoresis of this fraction using 7.5 % polyacrylamide gel showed only one major band which was stained blue with Coomassie blue. When gels were run in parallel but stained differently (alcian blue for glycoproteins, periodic acid-Schiff for carbohydrate, and Sudan black B for lipid), a single band was again observed in each gel at mobilities corresponding to that of the protein band (Fig. 12).

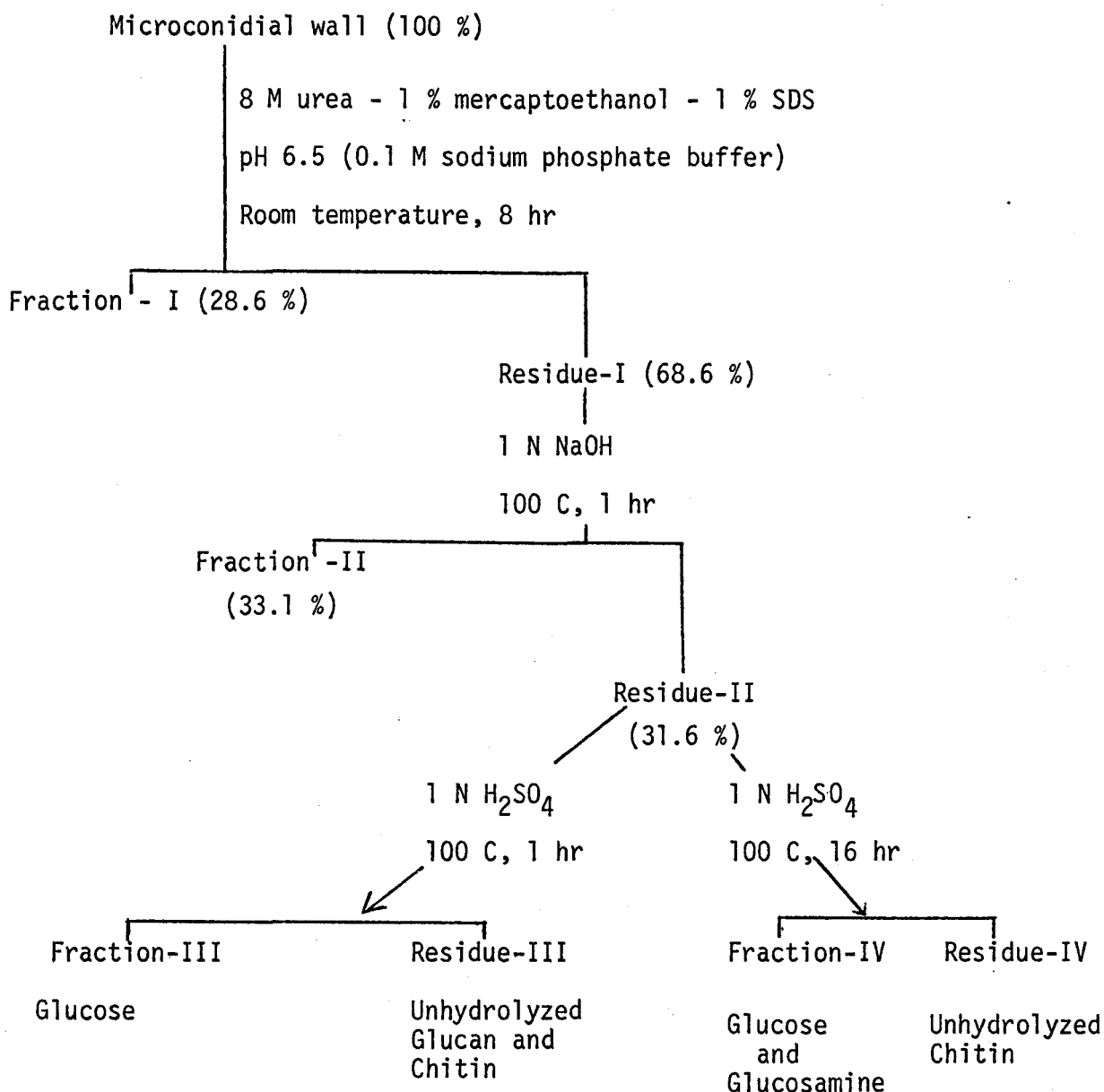
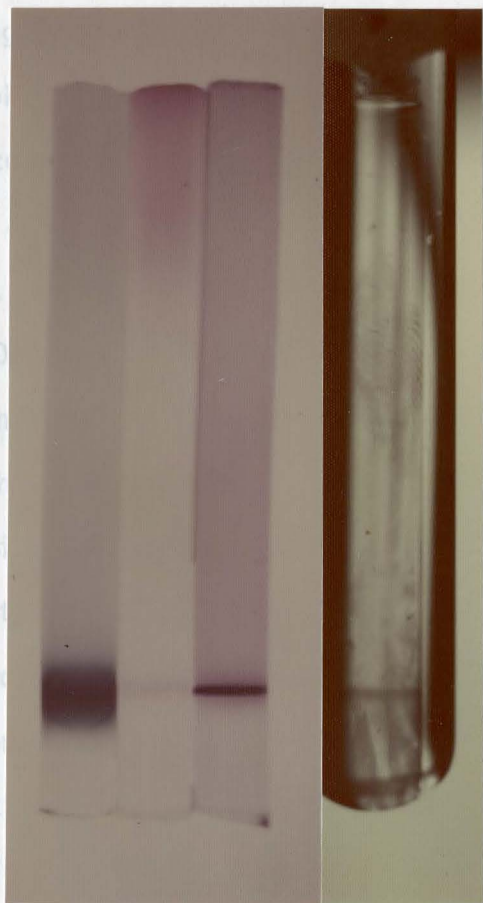


Fig. 11. Distribution of fractions of wall polymers obtained from chemical extractions of microconidial wall of T. mentagrophytes.^a

^a Microconidial walls of T. mentagrophytes were extracted with chemicals using procedures described in Fig. 1. Values in parenthesis represent per cent dry weight of intact microconidial wall.

Fig. 12. Disc gel electrophoresis(using 7.5 % polyacrylamide gel) of fraction-I isolated from microconidial walls by the UMS treatment. From left to right: gel stained with alcian blue for glycoprotein, gel stained with periodic acid-Schiff reagent, gel stained with Coomasie blue for protein, and gel stained with sudan black B for lipid.



This indicated that fraction-I could be a protein-carbohydrate-lipid complex.

When gels were run at different pH values, different migration patterns were observed. At pH 8.3, the band migrated toward the anode; at pH 7.5, no migration was seen; and at pH 6.6, the reversed polarity of migration took place. It seemed likely that the isoelectric point of the protein component resided between pH 6.6 to pH 7.5. However, when fraction-I along with other known molecular size proteins such as: hemoglobin (M.W. 60,000), human serum albumin (M.W. 67,000), and human IgG (M.W. 160,000), were run on gradient gels, no bands were observed upon staining when the smallest protein standard (hemoglobin; 60,000 M.W.) reached the end of the tube. This showed that the protein component of fraction-I, besides its acidic nature, had a faster electrophoretic mobility than that of hemoglobin (60,000 M.W.).

Paper chromatography of the acid hydrolysate of this fraction revealed two monosaccharide spots which were identified as glucose and mannose at a ratio of 2 : 1. No amino sugar was detected.

Amino acid composition of this fraction is shown in Table 5. It is characterized by its high content of glutamic acid (11.56 %) and aspartic acid (10.66 %) in conjunction with sufficient amounts of NH_3 and 10 % basic amino acids, indicating the neutral nature of the protein component of F-I. This is consistent with the electrophoretic data described above.

Table 5. Amino acid compositions of fraction I extracted from microconidial walls by 8M urea - 1% mercaptoethanol - 1% SDS (pH 6.5, 0.1M sodium phosphate buffer).

Amino acid	% μ moles
Alanine	9.31
Arginine	4.60
Aspartic acid	10.66
Cysteic acid	0.20
Glutamic acid	11.56
Glycine	9.87
Half cystine	0.78
Histine	tr
Isoleucine	4.26
Leucine	7.29
Lysine	5.49
Methionine	1.12
Phenylalanine	3.59
Proline	6.50
Serine	9.53
Threonine	6.73
Tyrosine	2.69
Valine	5.72

Examination of fraction-I for possible enzymic function showed that this component had no proteolytic activity against protein substrates such as albumin or casein. When freshly harvested microconidia were incubated with either of the protein substrates (at 37 C for 30 min up to 18 hr), no cell associated proteolytic activity was detected.

R-I residue. As shown in Fig. 13b, the microconidial walls extracted by UMS (residue R-I) retained their original shape. When sectioned and examined under the electron microscope (Fig. 14), the UMS treatment appeared to remove a bulk portion of the electron dense layer of the outer wall, especially the outer filmy layer (OW1). The OW2 layer, however, remained undisturbed. No alteration in the ultrastructural morphology of the inner wall (IW) layer was observed. The appearance of the microfibrils on the inner surface of the walls appeared similar to that of the intact, untreated walls, and was not affected by such treatment (data not shown). The UMS treatment does not seem to affect significantly the rodlet layer (data not shown).

Chemical analysis of the R-I residue showed the presence of 64.1% carbohydrate, 5.2 % protein, and 22.5 % chitin (Table 6). Sugars identified by paper chromatography in the acid hydrolysate of this fraction were glucose, mannose, and glucosamine, with trace amount of galactose.

2. Hot alkali extraction of the UMS treated microconidial walls.

Since the UMS extraction appeared to have removed one component from the intact microconidial wall, subsequent effort was made to

Fig. 13. Phase contrast photomicrographs of microconidial walls after various chemical extractions. a, intact microconidial walls; b, microconidial walls treated with UMS, c, microconidial walls treated with UMS followed by hot alkali (1 N NaOH at 100 C for 1 hr); and d, microconidial walls treated with UMS followed by hot alkali (1 N NaOH at 100 C for 1 hr) and mild acid (1 N sulfuric acid at 100 C for 1 hr) treatment.

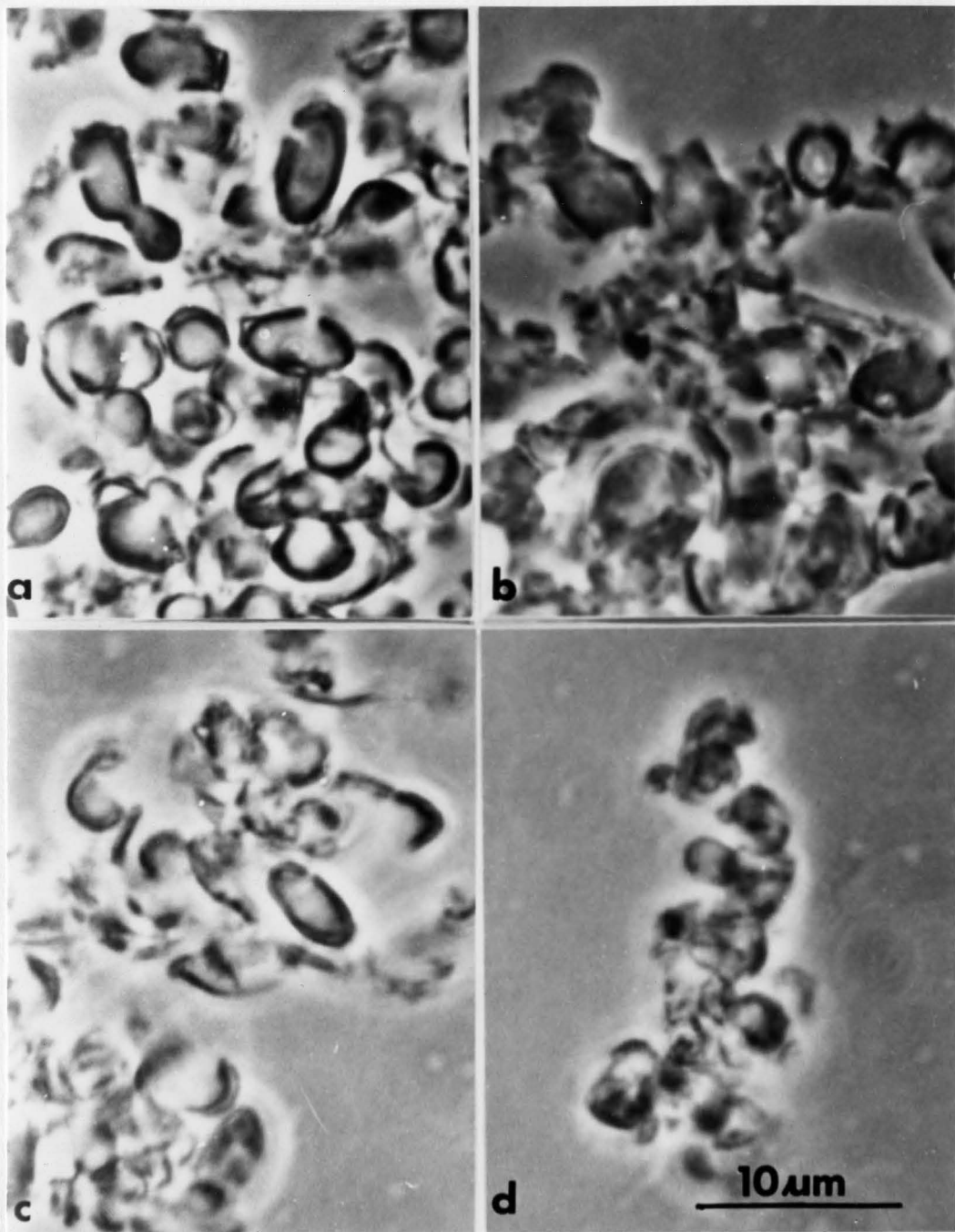


Fig. 14. Electron micrograph of an ultrathin section of the UMS extracted microconidial walls. Note that this treatment removed a bulk portion of the outer wall layer while the second electron dense layer (arrow) remained discernible.

Table 6. Chemical composition of the UMS extracted microcompartments
walls of T. mentagrophytes.^a

Component

% Dry weight^b

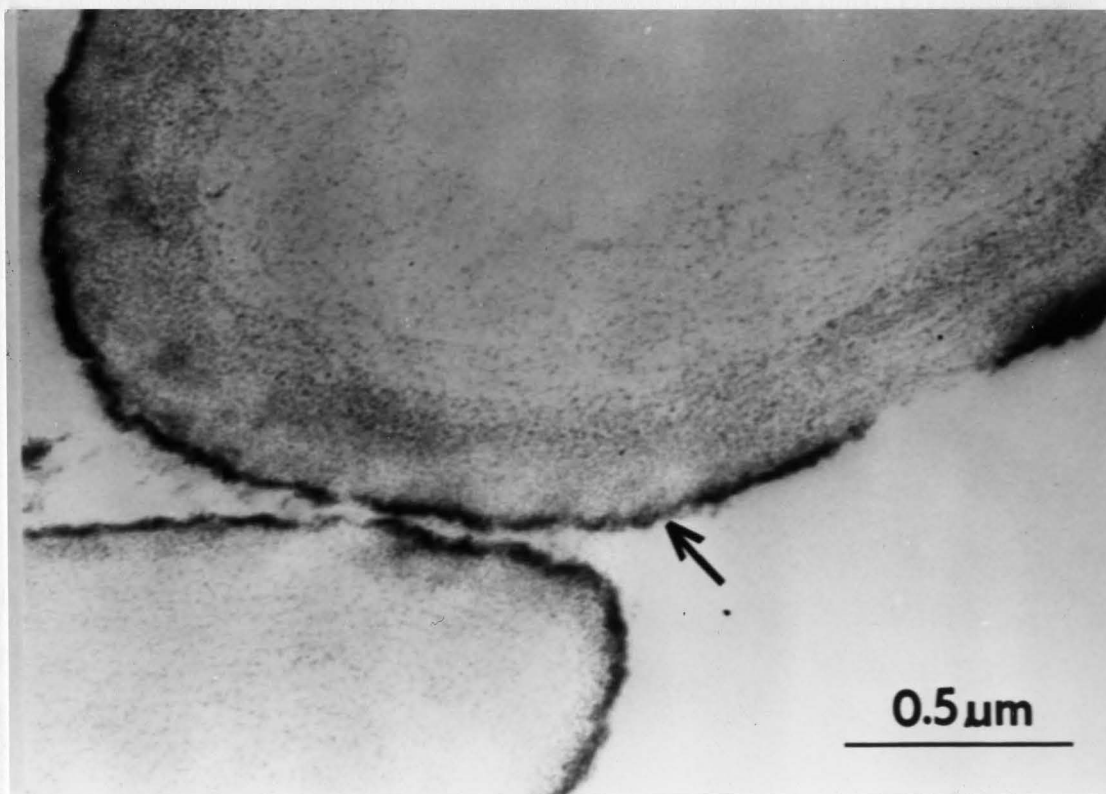


Table 6. Chemical composition of the UMS extracted microconidial walls of T.mentagrophytes.^a

Component	% Dry weight ^b
Carbohydrate (as glucose)	64.1
Protein	5.2
Chitin	22.5

^a T.mentagrophytes microconidial walls were extracted with 0.1 M sodium phosphate buffer (pH 6.5) containing 8 M urea-1 % mercaptoethanol-1 % SDS at room temperature for 8 hr. The wall residue (R-I) was lyophilized and characterized chemically using methods described previously (see Materials and Methods section).

^b Values represent percent dry weight of wall residue (R-I).

isolate and characterize the remaining wall polymer(s) that resisted the UMS treatment. For this purpose, UMS extracted microconidial walls were treated further with acid, alkali, or organic solvents under different conditions. Freeze etched preparations of each extracted wall residues were examined under the electron microscope. As it is evident in Table 7, boiling with 1 N NaOH for 1 hr at 100 C after the UMS extraction caused a remarkable alteration of the surface rodlet structure. The characteristic pattern previously observed on the wall surface was lost by the hot alkali treatment. After such a treatment, the disorientation and dissolution of the rodlets became apparent (Fig. 15). Therefore, a material or materials extracted from the UMS treated microconidial walls by hot alkali was subsequently characterized.

Fraction II. The hot alkali extract of the UMS treated walls was mixed with two to three volumes of cold ethanol (to a final concentration of 66-70 %), and the precipitate thus obtained was referred to as fraction II. This fraction, after washing with cold ethanol, accounted for 33.1 % dry weight of the intact microconidial walls (Fig. 11). It appeared to be light brown or yellowish in color, viscous, and readily soluble in water at room temperature. Examination of the ultraviolet spectra of both the hot alkali soluble, ethanol precipitable and non-precipitable fractions showed no detectable peaks at 260 or 280 nm. Quantitative determination of protein using the Lowry method showed negligible amount of protein present (less than 1.5 %).

Table 7. Effect of some chemical extraction procedures on the rodlet layer of T. mentagrophytes microconidial wall.

Extraction condition	Solubilization of rodlets ^a
UMS (4 M) ^b , room temperature, 2 hr	
+ 1 N NaOH, room temperature, 5 hr	-
UMS (4 M), room temperature, 2 hr	
+ 1 N NaOH, 50 C, 5 hr	-
UMS (4 M), room temperature, 2 hr	
+ 1 N sulfuric acid, room temperature, 5 hr	-
UMS (4 M), room temperature, 2 hr	
+ 1 N sulfuric acid, 50 C, 5 hr	-
UMS (4 M), room temperature, 2 hr	
+ 95 % ethanol, room temperature, 1 hr	-
UMS (4 M), room temperature, 2 hr	
+ chloroform, room temperature, 3 hr	-
UMS (8 M) ^c , room temperature, 8 hr	
+ 1 N NaOH, 37 C, 1 hr	-
UMS (8 M), room temperature, 8 hr	
+ 1 N NaOH, 50 C, 1 hr	-
UMS (8 M), room temperature, 8 hr	
+ 1 N NaOH, 100 C, 1 hr	+
UMS (8 M), room temperature, 8 hr	
+ 1 N H ₂ SO ₄ , 100 C, 1 hr	-

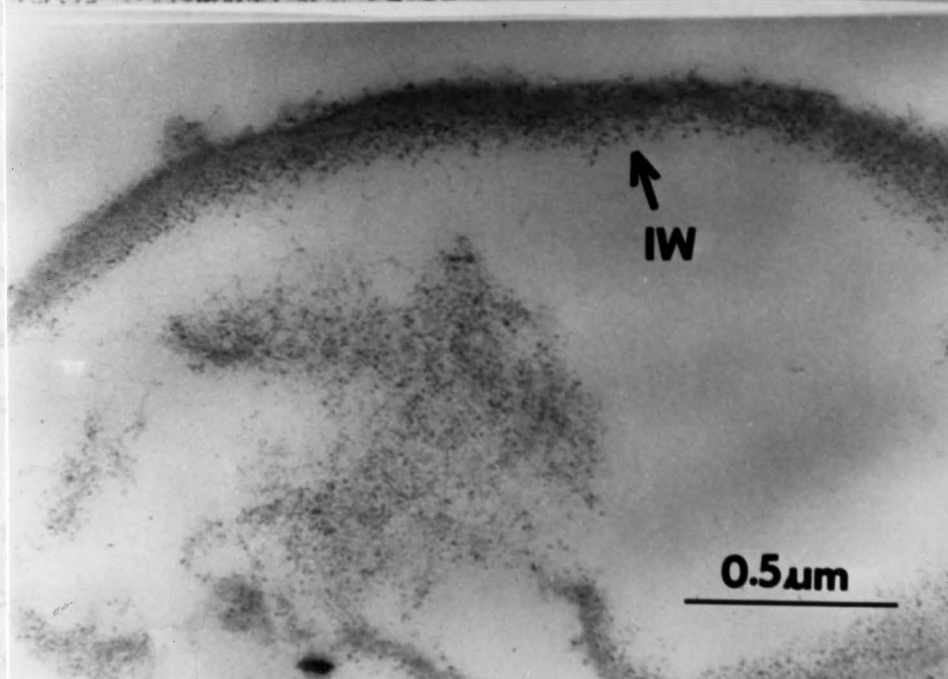
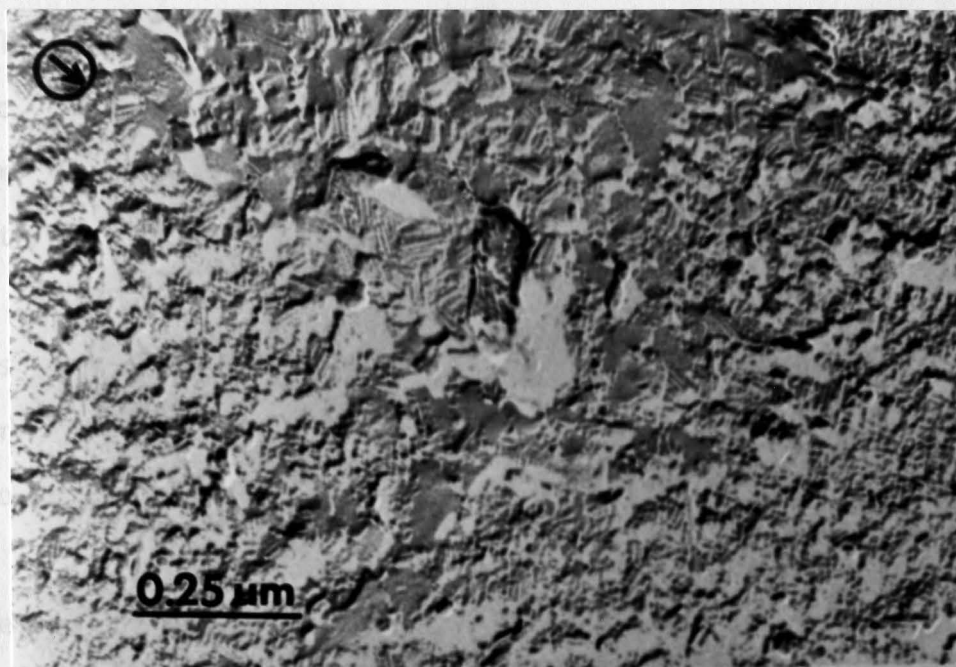
Table 7. (Contd.)

- a T. mentagrophytes microconidial walls were extracted with various chemicals (as listed in the Table) under specified conditions. Freeze-etched preparations of conidial wall residues after each treatment were made and examined under the electron microscope. "+" represents the dissolution of rodlet layer, while "-" represents no change in morphology of the rodlet layer.
- b "UMS (4 M)" represents 0.1 M sodium phosphate buffer (pH 6.5) containing 4 M urea, 1 % mercaptoethanol and 1 % SDS.
- c "UMS (8 M)" represents 0.1 M sodium phosphate buffer (pH 6.5) containing 8 M urea, 1 % mercaptoethanol, and 1 % SDS.

Fig. 15. Electron micrograph of a freeze-etched preparation of microconidial walls after UMS treatment and hot alkali treatment. Note the apparent alteration in the appearance of the rodlets. They no longer displayed the regular tightly woven structure observed on the intact wall surface. The dissolution and disorientation of the rodlets occurred.

Fig. 16. Electron micrograph of a thin section preparation of microdonidial walls extracted with UMS followed by hot alkali treatment. Note that the electron dense layer of the outer wall (OW2) previously present on the UMS treated walls was removed, and only the inner wall (IW) of lower electron density remained undisturbed.

Since polysaccharide may be the major component of F-II, analysis



The R-II residue accounted for approximately 31.6 % of the total

Since polysaccharide may be the major component of F-II, analytical tests for carbohydrate were carried out. Positive results obtained from both Molisch and anthrone tests indicated the presence of some carbohydrate materials. However, the amount detectable by the anthrone method using glucose as standard showed a low value of 10 %.

After hydrolysis with 1 N H_2SO_4 for 5 hr at 110 C, acid hydrolysate of F-II was spotted on paper chromatogram. Glucose and mannose were the only two neutral sugars detected in approximately equimolar amounts. No glucosamine was found in this fraction.

R-II residue. As described previously, electron microscopy of the freeze-etched surface of the microconidial walls treated with UMS and hot alkali (R-II residue) revealed the dissolution of the rodlet patches (Fig. 15). As shown in an electron micrograph of thin section of the wall residue (Fig. 16), the hot alkali treatment apparently removed one of the outer electron dense layers (OW2) of the microconidial wall. The thick inner wall of low electron density (IW) does not seem to be affected significantly by hot alkali treatment. Although not shown here, electron microscopy of the shadowed preparation of the R-II residue still displayed the microfibrillar structure on the inner surface layer. When examined under the dark phase contrast microscope, the residual walls still retained the original shape but appeared somewhat thinner than the untreated (Fig. 13c).

The R-II residue accounted for approximately 31.6 % of the intact wall dry weight (Fig. 11). When hydrolyzed with 1 N H_2SO_4 at 110 C for 16 hr, glucose and glucosamine were detected on the paper chromatogram.

The presence of glucosamine was resulted from partial hydrolysis of chitin. The carbohydrate content estimated by the anthrone method was 55.6 % (percent dry weight of R-II, as glucose). Chitin as determined by the method of Blumenthal and Roseman (1957) gave a value of 39.7 % which accounted for the rest of the R-II residue. Protein only represented a minor portion of this residue which was 3.7 % (percent dry weight of R-II) (Table 8). These data suggested that the UMS and hot alkali treated walls were composed of a glucose polymer which was alkali-insoluble, and an N-acetyl glucosamine polymer (chitin).

Microconidial walls extracted with hot alkali without prior UMS treatment contained galactose, glucose, mannose, and glucosamine (Table 9). However, walls treated first with UMS and then hot alkali contained only glucose and glucosamine (Table 9). Hot alkali treatment of the UMS extracted walls appeared to be the only condition that caused both the dissolution of the rodlets and the release of galactose, mannose and some glucose from R-I.

3. Acid extraction of the UMS + alkali treated microconidial walls (R-II).

a. Mild acid treatment of R-II.

Fraction-III. As described previously, the R-II fraction consisted of glucan(s) and chitin. The mild acid hydrolysate (1 N sulfuric acid at 110 C for 1 hr) contained glucose only (F-III).

Residue-III. Further treatment of R-II residue (UMS + hot alkali treated walls) with mild acid resulted in the removal of most of the glucan. Electron microscopic examination of the

Table 8. Chemical composition of wall residue (R-II) after UMS and hot alkali extraction^a.

Component	% Dry weight ^b
Carbohydrate (as glucose)	55.6
Protein	3.9
Chitin	39.7

^a The UMS extracted walls of T. mentagrophytes microconidia (R-I) were treated with 1 N NaOH at 100 C for 1 hr and the insoluble wall residue (R-II) was thoroughly washed and dried.

^b Values represent percent dry weight of wall residue (R-II).

Table 9. The sugar composition of I. mentagrophytes microconidial walls extracted by UMS and alkali^a.

Extraction condition	Sugar composition of the extracted walls
UMS ^b (8 hr)	galactose, glucose, mannose, and glucosamine
UMS (12, and 16 hr)	galactose, glucose, mannose, and glucosamine
UMS (8, 12, and 16 hr) followed by 1 N NaOH at 100 C for 1 hr	glucose and glucosamine
1 N NaOH at 100 C for 1 hr	galactose, glucose, mannose, and glucosamine

^a Microconidial walls of I. mentagrophytes were extracted with chemicals under various conditions listed in the Table. Extracted wall residues were hydrolyzed with 1 N sulfuric acid at 110 C for 16 hr, and monosaccharides in hydrolysates were identified by paper chromatography.

^b 8 M urea-1 % mercaptoethanol-1 % SDS at room temperature (pH 6.5; 0.1 M sodium phosphate buffer).

shadowed residue (R-III) revealed the presence of aggregates of microfibrillar materials oriented more or less parallel to each other (Fig. 17). Phase contrast microscopic examination of R-III showed that these walls still retained their original shape (Fig. 13d).

Paper chromatography of the acid hydrolysate of R-III showed the presence of glucosamine and glucose. Enzymatic digestion of this residue with (1 \rightarrow 3)- β -D-glucanase and chitinase released glucose and N-acetyl glucosamine respectively (Hashimoto, personal communication, 1975).

b. Extensive acid treatment of R-II.

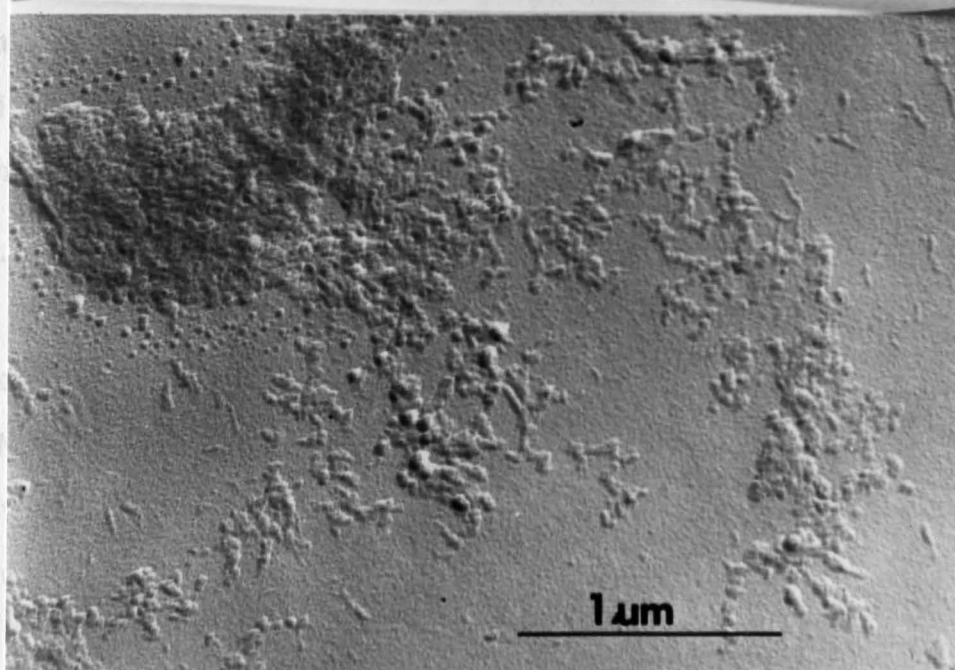
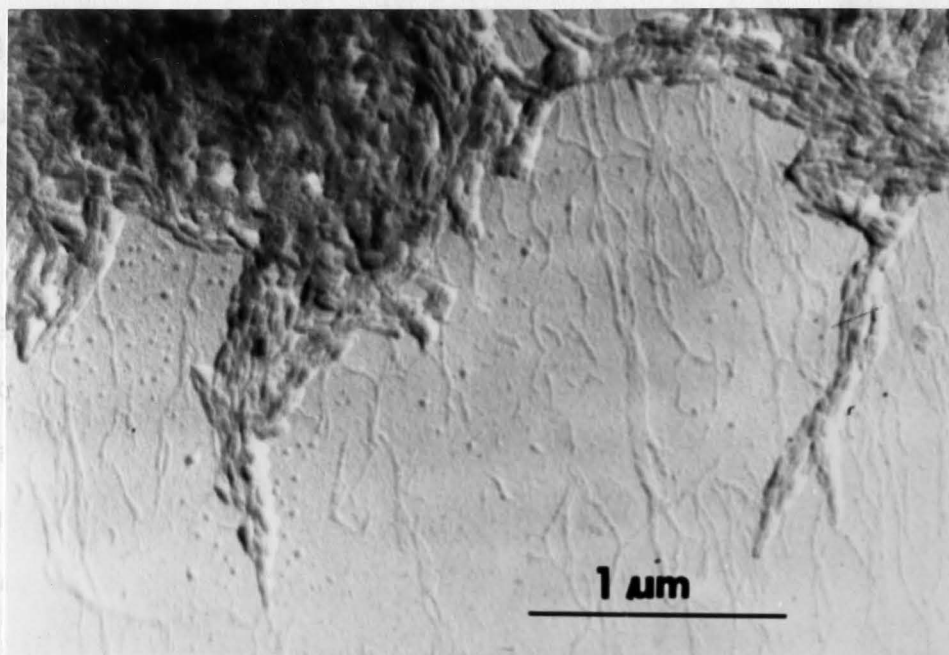
Fraction IV. The R-II was more extensively hydrolysed in 1 N sulfuric acid at 110 C for 16 hr. This treatment effectively removed essentially all of the glucan matrix that was not completely hydrolyzed by the mild acid treatment. Paper chromatography of the acid hydrolysate showed the presence of glucose and glucosamine. Chitin in R-II were hydrolyzed by drastic acid treatment for prolonged periods of time.

Residue-IV. This fraction represented the residue of the microconidial wall resistant to UMS, hot alkali and hot acid (1 N H₂SO₄, 110 C for 16 hr) extractions. Electron micrograph of the shadowed preparation of this fraction (Fig. 18) revealed that the matrix material was completely removed along with the disintegration of some chitin fibers. Although not shown here, phase contrast microscopic examination of this fraction showed the presence of small debris. The shape of the intact walls was no longer retained.

Fig. 17. Electron micrograph of a shadowed preparation of microconidial wall treated with UMS, hot alkali, and mild acid. Microconidial walls were extracted with UMS, and hot alkali, followed by mild acid hydrolysis in 1 N sulfuric acid at 100 C for 1 hr. The wall residue was shadowed with carbon-platinum and examined with the electron microscope as described previously. Note that the acid treatment removed most of the matrix material formerly present on the inner wall surface. The thicker fibers arranged in the characteristic bundles were apparent.

Fig. 18. Electron micrograph of a shadowed preparation of microconidial walls treated with UMS, hot alkali, and extensive acid hydrolysis. Microconidial walls were extracted with UMS, and hot alkali, and followed by extensive acid hydrolysis (1 N sulfuric acid at 110 C for 16 hr). The residual walls were shadowed with carbon-platinum and examined under the electron microscope. The removal of all of the glucan matrix along with most of the chitin fibers was observed. However, fragments of chitin fibers could occasionally be seen in the background, probably due to incomplete hydrolysis.

Chemical analysis of R-IV showed the presence of glucosamine



Microconidial walls were first digested with glucosylase

at 37°C. After thorough washing, the wall residues were

Chemical analysis of R-IV showed the presence of glucosamine only. When incubated with chitinase, residue IV gave rise to N-acetyl glucosamine.

D. Enzymatic digestion of the microconidial walls.

Purified microconidial walls were incubated with prefiltered snail digestive enzyme (glusulase) for 48 hr at room temperature. Electron microscopy of ultrathin sections of glusulase treated walls revealed that the inner wall layer (IW) was completely digested leaving a layer comparable to one of the outer layers of the intact walls (Fig. 19). When a freeze-etched preparation of the glusulase treated walls was examined with the electron microscope, the rodlets similar to those shown in Fig. 9 were found to remain intact. Under the phase contrast microscope, the enzyme treated wall residue retained their original shape (Fig. 20b).

E. Preliminary characterization of the rodlet layer.

As described in the above section, the glusulase treated microconidial walls still retained the rodlet layer on their surface. Since UMS treatment was shown to remove the outer filmy layer without affecting the rodlets, it raised a possibility that the rodlet layer could be isolated in a pure form for further chemical characterization.

1. Preparation of the rodlet layer.

Microconidial walls were first digested with glusulase for 48 hr at room temperature. After thorough washing, the wall residues were extracted by UMS at room temperature for 24 hr. The resultant residue represented a crude rodlet layer preparation. A further purification

Fig. 19. Electron micrograph of an ultrathin section of the snail enzyme (glusulase) digested microconidial wall demonstrating the complete removal of the inner, electron lucid wall layer. The electron dense outer wall still exhibited the double-layered structure of the intact walls.

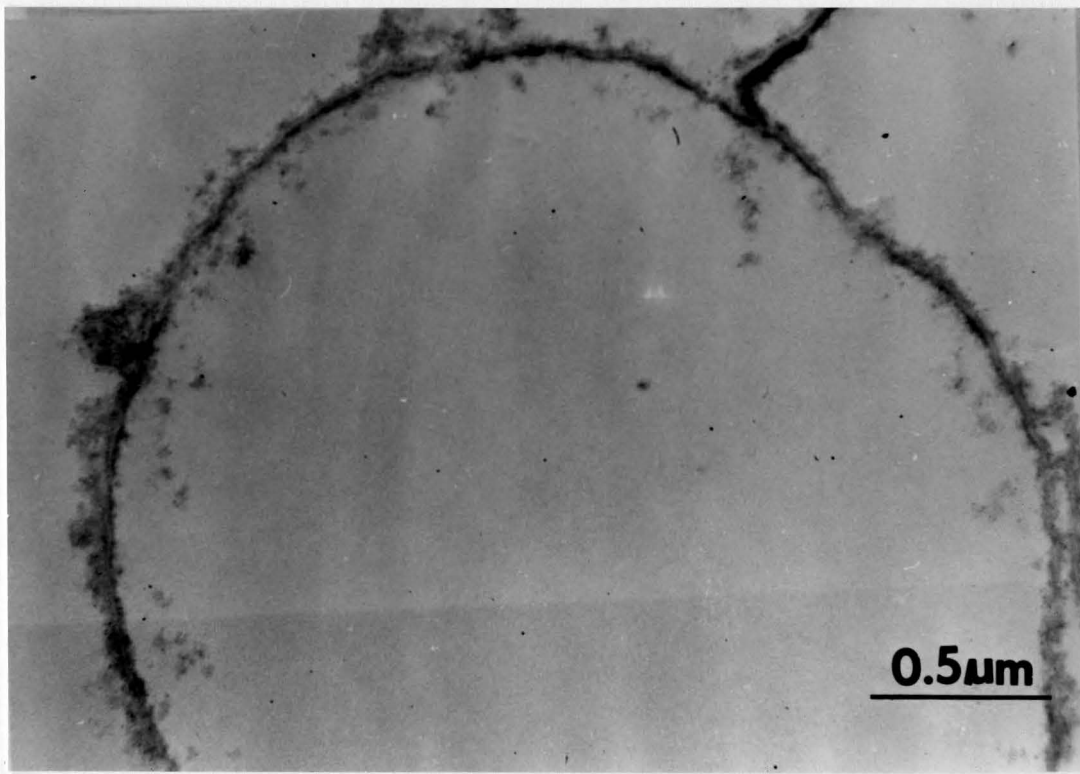
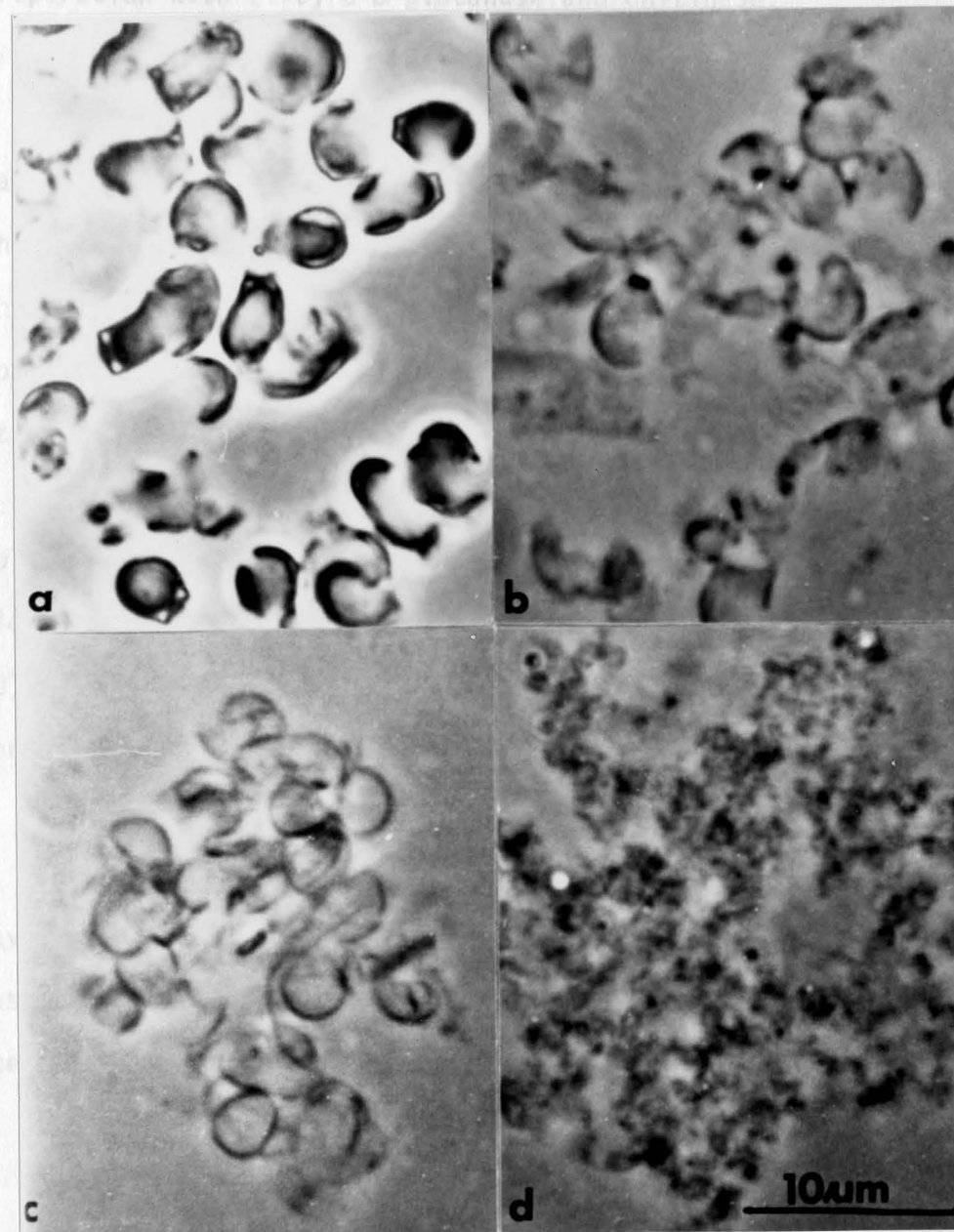


Fig. 20. Phase contrast photomicrographs of intact microconidial walls of *T. mentagrophytes* treated with enzyme and/or chemicals. a, intact microconidial walls of *T. mentagrophytes*; b, microconidial walls digested by glusulase at room temperature for 24 hr; c, microconidial walls treated with UMS followed by glusulase digestion at room temperature for 24 hr; and d, microconidial walls treated with UMS, glusulase followed by hot alkali (1 N NaOH, 100 C) treatment for 1 hr.



of the rodlet layers was achieved by additional treatments of this preparation with (1 \rightarrow 3)- β -D-glucanase and chitinase.

2. Morphology and physical properties of the rodlet layer.

The rodlet layer obtained above was light brown in color and was hydrophobic. As shown previously, the electron micrograph of the thin section prepared from glucanase digested walls consisted of a thin, double-layered structure (Fig. 19). When viewed under a phase contrast microscope, the rodlet layer appeared as thin flakes, while still retaining the original shape of the intact walls (Fig. 20c).

The isolated rodlet layer was not soluble in acids or organic solvents. Treatment of this layer with various chemicals or solvents did not cause the disintegration or dissolution of the structure (Table 10). It was found that only boiling in hot alkali (1 N NaOH at 100 C for 1 hr) resulted in the disintegration of the rodlet layer (Fig. 20d).

3. Chemical composition of the rodlet layer.

Paper chromatography of an acid hydrolysate of the rodlet layer showed no detectable sugar spots. The presence of carbohydrate was almost negligible as determined by the anthrone method. At present, the chemical composition of this unique structure is still unknown.

Table 10. Effect of chemicals, organic solvents, and enzyme on the integrity of the UMS and gluculase treated microconidial walls.^a

Condition	Disintegration of the UMS and gluculase treated wall
1 N H ₂ SO ₄ , room temperature, 1 hr	-
1 N H ₂ SO ₄ , boiling, 1 hr	-
1 N NaOH, room temperature, 1 hr	-
1 N NaOH, room temperature, room temperature overnight	-
1 N NaOH, 50 C, 1 hr	-
1 N NaOH, 50 C, 5 hr	-
1 N NaOH 100 C, 1 hr	+ ^b
Chloroform and methanol (2:1), room temperature, 1 hr	-
Chloroform and methanol (2:1), room temperature, overnight	-
Lysozyme (pH 7.0), room temperature, 1 hr	-
Lysozyme (pH 7.0), room temperature, overnight	-
Water, boiling, 1 hr	-

^a Intact conidial walls, after extracted with UMS, were incubated with gluculase at room temperature for 24 hr. The wall residue, after thorough washings, was then subjected to various treatments with chemicals, organic solvents or enzyme. The effects of such treatments on wall morphology were examined under a phase contrast microscope. "+" represents disintegration of the UMS and gluculase treated walls, while "-" represents no alteration in wall integrity.

^b Phase micrograph of the UMS and gluculase treated walls subjected to subsequent alkali boiling was shown in Fig. 20c.

CHAPTER IV

DISCUSSION

Although there have been several studies on the chemistry and morphology of the hyphal walls of dermatophytes (Tsukahara et al., 1964; Pock-Steen and Kobayasi, 1970; Noguchi et al., 1975; and Poulain et al., 1975), there are no reports of similar studies of the microconidial walls. This could be due to the difficulties involved in purification and isolation of the microconidia and their walls. As is known, two types of conidia are produced by T. mentagrophytes: the microconidia and the macroconidia. The former are formed abundantly when the fungus is grown on agar surface. During the course of harvesting these microconidia from an agar surface, care must be taken to avoid contamination by macroconidia or vegetative hyphae. A method for the isolation and purification of the microconidia has been described (Hashimoto et al., 1972). These spore preparations were then subjected to mechanical disruption to obtain wall fragments.

A second difficulty associated with this study was the isolation of microconidial walls free from cytoplasmic contamination. The purity of a wall preparation usually being defined in terms of the absence of cytoplasm. This is determined commonly by phase contrast or dark field light microscopy, and by the use of stains such as cotton blue or methylene blue. When rigorous quantitative analysis of fungal walls is desired, the presence of contamination which is visible only under the electron microscope must be avoided. Chemical tests for purity involve

the identification of common contaminants such as nucleic acids or proteins. Nevertheless, a number of workers have found nucleic acids in preparations which they accepted as pure (Bartnicki-Garcia and Nickerson, 1962; and Horikoshi and Iida, 1964). In our study, the use of a Mickle cell disintegrator instead of the conventional alkali treatment for wall isolation provided chemically intact, clean wall material for both chemical and morphological studies. No distortion or alteration in structural characteristics of the microconidial walls were observed after this treatment (Fig. 5c). Most of the previously mentioned tests for wall purity were employed and the use of acridine orange stain was an additional agent successfully used for this purpose. It may be impossible to state unequivocally that our wall preparations were entirely free from cytoplasmic material, but evidence obtained from our data showed that such contamination was negligible.

This study has employed a combination of chemical extractions and analyses, and ultrastructural examination to develop a model of the microconidial wall of I. mentagrophytes. We are not aware of a previous report of such work. In the past, examination of thin sections, shadowed preparations, or replicas of freeze-etched preparations of walls have been used for the study of the ultrastructure of cell walls. No one technique can sufficiently provide the accurate or detailed information sufficiently needed for such a study. Thin sections often provide limited information on the actual ultrastructure of electron-dense or electron translucent material in the cell envelope. Shadowed preparations may reveal the architecture within a particular layer, but

the structure observed in the shadowed specimen of chemically treated walls often deviates from the true position in the intact cell. The special characteristics of the freeze-etching technique allow the observation of more unaltered structural detail than either thin sectioning or shadowing. A three-dimensional image of the walls can best be derived when data obtained from sectioned material, shadowed wall fragments, and freeze-etched wall surfaces are integrated. However, the identification of individual macromolecular cell wall components is not possible using the electron microscope alone. No information can be provided as to how the different polymeric constituents contribute to the image. Through the use of specific sequences and combination of chemical and/or enzymatic attacks on microconidial walls (Fig. 1), it was possible to isolate and characterize individual wall fractions systemically. The chemical nature of the individual wall fractions could then be correlated to the morphologically distinct layers, and some conclusions on the location and interaction of these polymers in the chemically intact microconidial wall drawn.

As reviewed in a previous section (Chapter I), there are numerous reports of the existence of more than one layer in spore walls of either yeast or filamentous fungi. In walls of *T. mentagrophytes* microconidia, two well defined layers were distinguished (Fig. 7). Although the chemical nature of this two-layered structure has never been reported, it resembled spore walls of many other filamentous fungi (Buckley et al., 1966; Campbell, 1969; and Carroll and Carroll, 1974), and consisted of outer electron dense layers and an inner layer of low electron density.

However, it was noticed that the density of the inner wall varies with the extent of fixation or staining. As shown in Fig. 5c, the well-stained isolated walls seemed to have a darker inner layer than that observed in the dormant microconidia (Fig. 7). At the same time, the double-layered structure of the outer wall was not as clearly resolved as that seen in Fig. 7. This indicated that when the inner walls were stained heavily with metal stain, the outer wall usually lost its distinct identity in thin sections. Therefore, care must be taken when one tries to interpret the electron micrographs. This problem reinforces the need to use a variety of preparative techniques when undertaking ultrastructural studies of a complex entity such as fungal spores.

Since there have been no previous reports on ultrastructural studies of walls of I. mentagrophytes microconidia or other dermatophyte spores, a comparison was made between our findings with that reported for walls of the vegetative hyphae. The appearance of the ultrathin sections of microconidial walls was somewhat similar to that of the vegetative walls. Pock-Steen and Kobayasi (1970), by examination of ultrathin sections, reported the double-layered wall structure of the vegetative hyphae of I. mentagrophytes. In shadowed preparations, the microconidial walls also resembled the vegetative walls in appearance. Noguchi et al. (1971) demonstrated the outer layer of the vegetative wall to be amorphous and the inner surface to be fibrillar. However, the rodlet layer observed on the microconidial wall surface has never been reported in studies of vegetative walls. Very recently, Hasegawa (1975) reported the presence of such a structure on freeze-etched

surface of I. mentagrophytes cell walls. Rodlets were seen to be present on the surface of older, aerial hyphae undergoing microconidiation (unpublished data). In a limited observation, no rodlet layer could be demonstrated by the freeze-etching technique on the surface of young hyphae growing in Sabouraud glucose broth.

Considerable effort was made in this study to determine optimal conditions for various chemical analyses because of the reasons stated earlier. Of primary importance was the condition for acid hydrolysis, an analytical procedure used throughout this study. As reported by Berst et al. (1969) and Dutton (1973), two important factors were involved during acid hydrolysis of polysaccharides: the release and the simultaneous degradation of the monosaccharides. As is evident from Table 1, the conditions used for fungal wall hydrolysis by different investigators has varied considerably. Further different conditions for hydrolysis have been used for studies of the cell walls of the same fungal species making it difficult, if not impossible, to compare results from various laboratories. Although the condition chosen for the hydrolysis of I. mentagrophytes microconidial walls (1 N sulfuric acid at 110 C for 16 hr) did not result in the complete release of the constituent sugar of chitin, it was found to have the least destructive effect of all conditions. Treatment of monosaccharides by this method resulted in no degradation, as shown by the paper chromatography studies (p. 59). Since no more than 5 % of sugar decomposed when heated in 1 N sulfuric acid at 110 C for 16 hr, subsequent values obtained from sugar quantitation were not adjusted for losses during hydrolysis. Due to

the presence of chitin in the walls, the release and decomposition of glucosamine during heating in acid for prolonged period of time was possible. Although Noguchi et al. (1974) reported that some glucosamine was decomposed when heated in hydrochloric acid, results in our study clearly indicated that sulfuric acid had no destructive effect on glucosamine during heating at high temperature. These data are in agreement with the earlier report by De Bruyne and Wouters-Leysen (1971) that hydrochloric acid causes more degradation than sulfuric acid.

Since the anthrone method for quantitation of sugars was used throughout this study, experiments were also undertaken to determine the optimal heating time for the procedure. It was known that when carbohydrates are heated in sulfuric acid with anthrone, a series of reactions, such as hydrolysis of polysaccharides to monosaccharides, degradation of monosaccharides, etc., occur (Toennies and Kolb, 1964). Previous workers (Morris, 1948; and Viles and Silverman, 1949) found that the color intensity of the anthrone reaction with carbohydrate was influenced by the heating time after addition of anthrone. However, variation in heating time from 10 to 20 min did not affect the color development of either sugar standards or microconidial walls with the anthrone reagent. Therefore, boiling time of 10 min with anthrone was considered optimal for the assay of total carbohydrate of microconidial walls.

It has been suggested that hydrolysis of carbohydrates with dilute acid before reaction with anthrone enhances the release of monosaccharides (Toennies and Kolb, 1964). However, prolonged incubation

of T. mentagrophytes microconidial walls with sulfuric acid (66 %) at room temperature for 10 to 30 min before the addition of anthrone did not increase the total glucose detected.

Paper chromatography of acid hydrolysates of various fractions or wall residues was used throughout this study as a means of separating and identifying constituent sugars. Although the solvent system and experimental conditions used provided excellent separation of individual sugars, repeated attempts to quantitate precisely the individual sugars on the chromatogram were not successful. Elution of the spots was attempted according to the method described by Chaykin (1966), but the technical error thus introduced made it impossible to obtain reproducible results. Therefore, the visual estimation of color intensities of sugar spots was used to obtain an estimation of the relative content of the monosaccharides from the various fractions.

Chemical analyses showed that the microconidial wall contained carbohydrate as the major wall component. Together with chitin and protein, it accounted for almost 100 % of the wall dry weight. Phosphorus, lipid and ash were minor components. Apparently, microconidial walls of T. mentagrophytes possess a chemical nature nearly as complicated as that of many other filamentous fungal spores.

The monosaccharide components of the microconidial wall were identified as galactose, glucose, and mannose at a ratio of trace:3:1, with glucose being the major one. Although galactose was consistently detected on paper chromatograms, the spot was faint, and hard to visualize. The use of gas-liquid chromatography proved to be successful

for the identification of this minor sugar component present in the wall hydrolysate (Fig. 10b).

Bartnicki-Garcia (1968) suggested a correlation between fungal morphology and cell wall composition and structure, with morphology being determined by composition. In this study, when the composition of microconidial walls and that of the vegetative hyphae were compared, some distinct differences were noted. Microconidial walls had physical properties which were distinct from those of the vegetative walls. Centrifugation in Renografin density gradients (Fig. 6) indicated that the microconidial walls were less dense than the vegetative walls. Chemically, the major components of vegetative walls were qualitatively similar to those of the microconidial walls. The greatest quantitative difference was in protein content, which was two times higher in microconidial walls. When amino acid composition of these proteins were analyzed, no qualitative differences in sulfur-containing amino acid (cystine), aspartic acid, asparagine, proline and tyrosine could be seen.

There are no previous findings on dermatophyte spore wall chemistry with which to compare the data of this study. However, the data observed upon analyses of vegetative walls (Table 3) differs from that of previous workers. Cummins and Harris (1958) could not detect the presence of galactose. Further studies of Noguchi et al. (1971) showed that glucose and mannose were the only two sugars in hyphal wall polysaccharides, and that they obtained a ratio of 3 to 1. In the most recent work carried out by Noguchi et al. (1975) on the carbohydrate

compositions of hyphal walls of thirteen dermatophyte species, a glucose to mannose ratio of 2.3 to 1 was reported for T. mentagrophytes. Galactose was not detected. However, our data clearly indicated that galactose was consistently detected in hydrolysates of vegetative and microconidial walls by both paper and gas-liquid chromatography. These discrepancies may be due to either the difference in methods used for fractionation and detection of sugar component, or to the difference of the age of the mycelial walls analyzed. The possibility that galactose was lost into the culture medium after prolonged periods of incubation cannot be ruled out.

Although our results obtained on the total protein content of vegetative walls (9.7 %) (Table 3) were comparable to those reported for walls of older (3 to 4 weeks old) hyphae (Noguchi et al., 1971), definite differences existed in the quantitative and qualitative distribution of individual amino acids recovered. For example, Noguchi et al. (1971) could not detect the presence of any proline or sulfur containing amino acids (cystine and methionine). They also reported a value for the content of tyrosine, isoleucine, and leucine in the four weeks old mycelial walls, which is twice that found in this study (Table 3).

The total glucosamine content (14.5 %) obtained from our analysis of 18 hr hyphal walls was one half that reported by Shah and Knight (1968) and by Noguchi et al. (1971) for the 4 day old or 4 week old mycelial walls. Since chitin exists as one of the major rigid structural components of hyphal walls, it is unlikely to be broken down and

then reutilized. The higher chitin content in old mycelial walls probably contributes to the differences encountered.

Several explanations can account for the discrepancies between data in this study and those of previous workers (Shah and Knight, 1968; and Noguchi et al., 1971). The wall preparation used for study by previous workers were made from broth grown (Sabouraud glucose broth) cultures of different ages, varying from 4 days to 4 weeks. It has been noted that T. mentagrophytes, when grown in broth culture with rigorous aeration; often forms a pellical around the culture flask which contains the growth of aerial hyphae and microconidia. Therefore, upon harvesting, a mixture of mycelial mats and spores would be obtained. If this mixture were then subjected to mechanical disruption, wall fragments of both hyphae and spores would be produced. This introduced considerable differences in the interpretation of data obtained. Despite this fact, none of the workers attempted to separate the two types of walls when carrying out chemical analyses. Another important factor contributing to the differences seen could be the age of the culture used for wall preparations. It is well known that the wall composition of fungi can vary with changes in growth conditions and culture age (Katz and Rosenberger, 1970). Considerable differences are known to exist in fungal wall composition depending on their growth stage (Bartnicki-Garcia and Reyes, 1964). In T. mentagrophytes, it was found that the total neutral sugars and glucosamine increased in parallel with the mycelial yield during the first few days of growth, and reached a maximum at fourteen to twenty days (Noguchi et al., 1975).

Using the fractionation procedures outlined in Fig. 1, it was possible to separate the microconidial wall into four major fractions: UMS soluble, fraction-I; UMS + hot alkali soluble, fraction-II; hot acid degradable, fraction-III; and acid and alkali resistant, fraction-IV. Each individual fraction was examined chemically and microscopically and could be discussed as a unique entity.

Although not discernible by the shadowing or freeze-etching techniques, electron microscopy of the ultrathin sections (Fig. 14) suggested that the UMS soluble fraction-I represented one of the electron dense layers located on the outermost surface of the walls. Extraction at room temperature for 8 hr proved to be sufficient for the maximal release of component-I from microconidial walls (p. 78), since no further release of wall components, either quantitatively or qualitatively, was observed when the extraction was prolonged. On the basis of data obtained from disc gel electrophoresis (Fig. 12) and chemical analyses, F-I is thought to be a homogeneous structural entity composed of a carbohydrate-protein-lipid complex.

Although the chemical data was insufficient to determine the exact structural configuration of this complex, some suggestions can be made on the carbohydrate moiety of this fraction. The fact that paper chromatographic analysis revealed glucose and mannose with a ratio of 2 to 1, and trace amount of galactose, suggests that the polysaccharide moiety is probably made of mainly a glucan with a mannan as branching molecules and possibly a terminal galactose.

It has been reported that surface protein or glycoprotein components present in fungal spore usually differed in amino acid composition from that of the whole spore wall (Gander, 1974; Page and Stock, 1974). The difference in amino acid composition between F-I and microconidial wall (Table 4 and Table 5) indicated that the protein in F-I exists as a different entity than the rest of the protein components present in the whole wall.

The existence of a mannan-protein complex has been found in cell walls of yeasts (Kessler and Nickerson, 1959; Korn and Northcote, 1960; and Kolarova et al., 1973). It has been reported that the carbohydrate component of the mannan is attached covalently to the protein or peptide-like material through a bond between N-acetyl glucosamine and asparagine, or by O-glucosidic bonds linked to the hydroxyamino acids (serine and threonine), in the peptide (Nakajima and Ballou, 1974). In bacterial cell walls, large amounts of glycine and alanine have been found and determined to provide cross-linkages within the peptidoglycan layer (Weber and Osborn, 1969).

Kitajima et al. (1972) removed an electron dense layer from hyphal walls of T. mentagrophytes by hot alkali extraction (1 N NaOH, 100 C for 5 hr). It was shown to be composed of mainly a galactomannan with 1,6- and 1,2-linkages, a β -glucan, and small amounts of peptides. It is likely that these constituents actually form a protein-carbohydrate complex within the outer electron dense layer of the vegetative walls similar to the F-I found in the microconidial walls. However, they did not detect the presence of such complex in the hot alkali extract.

UMS-soluble proteins have been reported as a component of the spores of several bacterial species, such as Bacillus thuringiensis (Delafield et al., 1968), B. cereus (Gould and Hitchins, 1963), and Clostridium roseum (Somerville et al., 1970). It usually accounted for 5 to 12 % of the dry weight of spores. In B. thuringiensis spores, the proteins were not required for their viability, nor were they involved with the resistance to heat or ultraviolet irradiation (Somerville et al., 1970). Gould and Hitchins (1963) reported that the extraction of protein by urea-mercaptoethanol increased the sensitivity of B. cereus spores to the action of lysozyme and also increased the rate of initiation of these spores during the germination process. In T. mentagrophytes, it was found that urea (2M)-mercaptoethanol(1 %)-SDS(1 %) extraction at room temperature (up to 4 hr) did not affect the viability of microconidia. However, it did cause a lag in the normal germination process (data not shown).

The release of a protein-lipopolysaccharide complex from isolated cell envelopes of gram negative bacteria by various chemical treatments has been reported (Royers et al., 1969). Recently, Narita and Manire (1976) isolated a protein-carbohydrate-lipid complex from Chlamydia psittaci by EDTA and alkaline buffer extraction. It consisted of 41.2 % carbohydrate, 8.9 % lipid, and 1.8 % protein. However, no specific roles were assigned to the isolated complex. Although derived from different origins and obtained from different extraction procedures, these protein-carbohydrate-lipid complexes might have similar functions for the organisms.

A number of enzymes in yeasts and filamentous fungi are found external to the cytoplasmic membrane and bound to certain wall components (Bartnicki-Garcia, 1966; and Ruiz-Herrera, 1967). Many of them have carbohydrate covalently attached to the peptide. Four glycoproteins were isolated from autolyzing Saccharomyces carlsbergensis and were reported to have proteolytic activities (Maddox and Hough, 1970). Three of the proteases contained mannose and glucose, and the ratio was unique to the enzyme. Although no effort was made to extensively characterize the enzyme activities of F-I, our data showed that this fraction contained essentially no proteolytic activities against protein substrates such as albumin or casein (p. 84). At present, it seems reasonable to assume that a complex comprising approximately 30 % of the microconidial wall should contribute significantly to some unknown properties of T. mentagrophytes microconidia. However, this merits further investigations.

The presence of a layer, on the microconidial surface, made up of tightly woven rodlets oriented at juxtaposition is evident from the electron micrograph (Fig. 9). The fact that the two electron dense layers (OW1 and OW2) remained intact after glucuronase digestion (Fig. 19) indicates that the rodlet layer, together with the UMS extractable filmy layer, composed the outer wall of the microconidia.

As revealed by the electron micrographs of carbon replicas and freeze-etched preparations, a network of double rodlets was observed on the surfaces of spores of some Bacillus species (Holt and Leadbetter, 1969), spores and aerial hyphae of Streptomyces species (Wildermuth et al., 1971), and conidia of several fungal species such as Penicillium

(Sassen et al., 1967) and Aspergillus species (Hess and Stocks, 1967). Hess and Stocks (1967) reported that in Aspergillus, when a freeze-etching technique was used, the resolution of the rodlet layer was increased. They thought that an additional layer might exist over the surface of the rodlet which accounted for the lack of resolution when the replica technique was used for the examination. Fisher and Richmond (1970) reported that in Penicillium conidia, a thin water soluble film of polyphosphate exists above the rodlets. Since the UMS soluble component (F-I) seemed to reside over the rodlet layer of T. mentagrophytes microconidia, it was thought that by removing the component, the rodlet layer might be visualized using the replica technique. However, extensive efforts by Hashimoto (personal communication) were unsuccessful in attempts to reveal the rodlet layer in carbon replicas, without prior freeze-etching, of microconidia treated by various extraction procedures, including: UMS, ethylenediamine, chloroform, sulfuric acid, sodium hydroxide, and glucuronidase treatments (unpublished data). Therefore, unlike the rodlet layers in spores of the above mentioned organisms, the rodlets on T. mentagrophytes microconidial surface could be revealed only by the freeze-etching technique.

Although rodlets are not generally well preserved by the thin sectioning technique, Ghiorse and Edwards (1973) demonstrated a striated outer wall surface of Aspergillus conidia, which they attributed to cross-sectioned rodlets. However, in our study, it was not possible to visualize the rodlets by such a method.

There has been no report of chemical analyses of ~~the~~ isolated rodlet layer. Bradley and Ritzi (1968) reported that in Streptomyces venezuelae, the rodlets disappeared from the spore surface after extraction with xylene, benzene, and ethyl alcohol. In I. mentagrophytes microconidia, the extensive extractions with various organic solvents did not remove the rodlet layer. These findings do not support the view that the surface rodlets of I. mentagrophytes were consistent of lipid.

Previously, Wessels et al. (1972) isolated a hot alkali soluble, crystalline layer from the hyphal walls of Schizophyllum commune, and reported that it was a glucan similar to the Schizosaccharomyces glucan (S-glucan). The fact that paper chromatography of acid hydrolysates of the rodlet layer isolated from I. mentagrophytes revealed no sugar spots indicates that this structure is not primarily made of carbohydrate. Moreover, the S-glucan differs from rodlets of I. mentagrophytes not only in its origin (hyphal wall rather than conidial wall), but also in appearance and enzyme susceptibility. The former was sensitive to glucanase digestion, while the latter resisted glucanase digestion and was much more tightly constructed.

A comparable structure found in eubacteria is the mosaic patterns of parallel bundles of rodlets described in the spore coat of some freeze-etched Bacillus species (Holt and Leadbetter, 1969). Although these rodlets are superficially similar in morphology, the rodlets in the bacterial spore coat consist of an alkali-soluble protein which can be removed by treatments which rupture disulfide bonds (Gould et al. 1970). In Penicillium expansum conidia, the rodlets were not removed

by incubation with mercaptoethanol in urea followed by alkali treatment (Fisher and Richmond, 1970). However, the rodlets were less distinctive after alkali treatment, suggesting that some material might have been removed. Analysis of the extracted material showed the presence of 11.2 % protein. They speculated that the remainder probably consisted of polysaccharides. No further effort has been reported concerning the identification of this material.

Recently, Kitajima and Nozawa (1975), by treating the walls with glusulase, isolated an enzyme-resistant exolayer from the vegetative hyphae of a dermatophyte, Epidermophyton floccosum. Chemical analyses of this layer showed that the main components were protein (63 %), mannose (10 %), and glucosamine (17 %). SDS gel electrophoresis revealed that the main band was a glycoprotein containing mannose, which had a different amino acid composition than the whole vegetative walls. It was recently found that the rodlets of I. mentagrophytes microconidia are composed mainly of protein and a trace amount of carbohydrate (unpublished data). The amino acid composition of this structure differs from that reported in the exolayer of E. floccosum (unpublished data). Moreover, the rodlets were not soluble in SDS or alkali. Although these two structures originate from different organisms, and vary in their chemical and physical properties, they seem to have two common features: they are both located on the outer surface of the walls, and they are both resistant to glusulase digestion.

Despite the fact that the chemical nature of the rodlet layer still needs further investigation, several hypotheses have been made

regarding the functions of this structure. Hydrophobicity, a characteristic of spores, is important for maximal spore dispersal. This characteristic has been thought to be due to properties of the spore surface. Fisher and Richmond (1970) thought that the rodlet layer was an integral part of the wall structure in Penicillium conidia, and contributed to the hydrophobic nature of the spore. Williams et al. (1972) found that spores of Streptomyces griseus became wettable upon removal of the rodlet sheath. The water-repellency of the rodlet layer of the I. mentagrophytes microconidia suggests that a similar situation exists.

A recent study by Hashimoto (personal communication) showed that the only common feature consistently observed along with the disintegration of rodlets (by hot alkali treatment, Fig. 20d) was the release of the light brown pigment into the alkali soluble fraction. It was subsequently found that this fraction contained a melanin-like pigment. In I. mentagrophytes, Kitajima et al. (1972) suggested the association of pigmented material with the outer electron dense layer of the hyphal walls. The solubilization of this pigment took place upon treatment with hot alkali (1 N NaOH at 100 C for 5 hr). However, the nature of this pigment was not characterized. Pigments such as melanin which can protect fungi from enzymatic lysis have been reported (Bull, 1970b). For example, in cell walls of wild type Aspergillus nidulans, melanin is responsible for its resistance to lytic enzymes (Bull, 1970a). It seems highly likely that a similar situation exists in I. mentagrophytes microconidia. However, the relation of the

melanin-like pigment to resistance of dermatophyte spores to enzymic lysis remains to be further investigated.

The fact that walls treated with UMS and hot alkali retained only the thick fibrous layer of the inner wall (IW) (Fig. 16), suggests that the inner layer (R-II) comprises the bulk of the microconidial wall and resides beneath the UMS-soluble component and the rodlet layer. The existence of this layer as a separate structural entity from the outer wall was clearly demonstrated by the enzymatic studies (Fig. 19). When intact microconidial walls were digested with glusulase, the outer wall remained (Fig. 19). The chemical and enzymatic studies together with electron microscopic examinations suggests that the inner wall is a layer composed of chitin fibers embedded in a glucan matrix. Digestion with glusulase completely degraded the inner wall and released glucose and glucosamine into the soluble fraction. A more specific enzymatic study using chitinase, and showing the release of N-acetyl glucosamine, confirmed the presence of chitin in the inner wall (Hashimoto, personal communication, 1975).

Data presented in Table 9 showed that the release of galactose from UMS extracted walls (R-I) took place after subsequent hot alkali treatment. However, galactose was not detected in either the hot alkali soluble fraction (F-II) or the residual walls (R-II) (Table 9). One possibility could be that during the extraction procedures, galactan was released but was too minute in quantity to be detected by the analytical method used in this study. Another possibility could be that a galactan or terminal galactose was lost during the fractionation procedures.

Mild acid hydrolysis of R-II removed the matrix material and released only glucose into fraction-III. Electron microscopic examination of shadowed fragments of the mild acid treated wall residue revealed the presence of aggregates of short microfibrillar particles (Fig. 17) similar to those reported by Jelsma and Kreger (1975). It was demonstrated in their study that a (1→3)- β -D-glucan, treated with hot acid, formed structures similar to those observed in our studies. This alkali-insoluble glucan differs from those of the outer wall layers (OW1 and OW2) in its glusulase susceptibility: the former was completely degraded upon glusulase treatment, while the alkali-soluble glucans of the wall were not susceptible to glusulase digestion. Recent data obtained by Hashimoto (personal communication) indicates that the linkages between the individual Trichophyton glucose monomers are β -1,3 linkages.

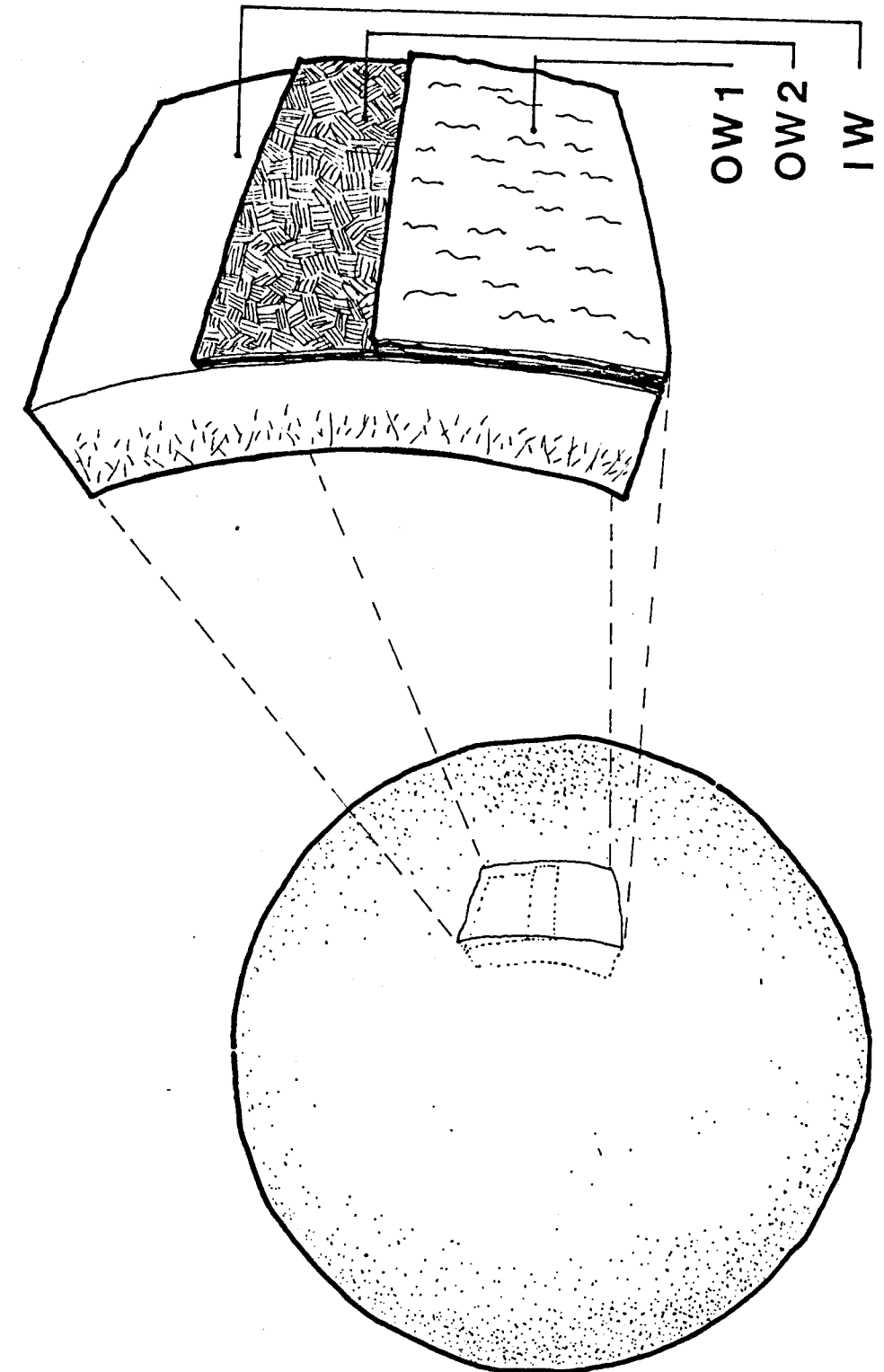
The presence of alkali-soluble glucans have been reported in the spore walls of several fungal species. Enzymatic digestion of Aspergillus oryzae conidial walls using (1→3)- β -D-glucanase indicated a preponderance of a β -1,3-linked, insoluble glucan (Horikoshi and Iida, 1964). Similar findings were reported for spores of Neurospora crassa (Mahadeven and Mahadka, 1970) and others (Bartnicki-Garcia, 1968). Although there are no other reports of insoluble glucans in microconidial walls of T. mentagrophytes, the results of this study can be compared with the results of studies of the hyphal walls. Kitajima et al. (1972) isolated a β -glucan with β -1,3 and -1,6 linkages from the hyphal wall of T. mentagrophytes using rather drastic alkali and acid

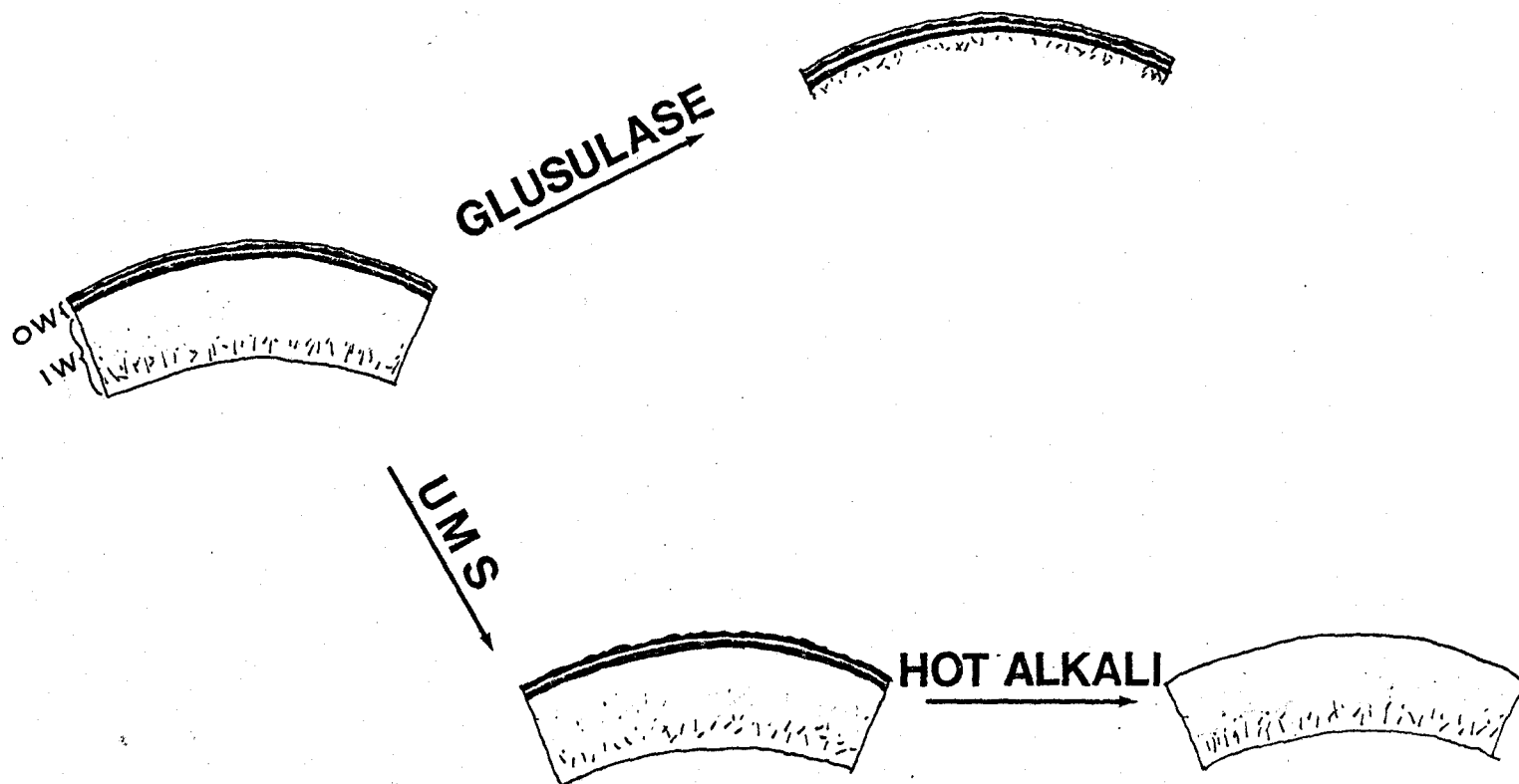
treatments. They did not report the presence of any alkali soluble glucan in these walls. It remains to be seen whether this reflects an intrinsic difference between hyphal and microconidial walls in the arrangement of the polymers, or is due to the different extraction procedures used.

In addition to the alkali-insoluble glucan, chitin is the other polymer remaining in the inner wall. Extensive acid hydrolysis of R-II (UMS + hot alkali treated microconidial walls) or digestion with gluculase, resulted in the disintegration of the walls (Fig. 18). During these treatments, N-acetyl glucosamine was released. From the characteristic solubility properties of this fraction: its alkali and acid resistance, and the above chemical and enzymatic studies, it can be concluded that the fibers cemented by a glucan matrix in the inner wall are composed of chitin.

On the basis of these studies, the structure of the microconidial walls of I. mentagrophytes is proposed (Fig. 21). The outer wall (OW) is composed of two thin, electron dense layers (OW1 and OW2). The outer layer (OW1) is readily extractable by UMS, while the inner electron dense layer (OW2), corresponding to the rodlet layer seen mechanically intact on the freeze-etched surface, is made up of material removable only upon hot alkali treatment. Under the outer wall, there lies the inner, thicker wall of low electron density (IW). This layer consists of an alkali-insoluble, (1 \rightarrow 3)- β -D-glucan, removable by acid hydrolysis, which cements the chitin fibers.

Fig. 21. A schematic model of the ultra architecture of wall layers of *T. mentagrophytes* microconidium. The outer wall (OW) is composed of two thin, electron dense layers (OW1 and OW2). The OW1 layer is readily extractable by UMS, while the OW2 layer, corresponding to the rodlet layer, is made up of material removable only upon hot alkali treatment. Beneath the outer wall (OW), there lies the inner thicker wall (IW) of low electron density. This layer consists of an alkali-insoluble, (1 \rightarrow 3)- β -D-glucan, removable by acid hydrolysis, which cements the chitin fibers.





It is a well known fact that the cell wall gives the fungal cell its characteristic rigidity and shape. However, little is known about the role of the individual polymers in these functions. Monocha and Colvin (1967) suggested that wall proteins may be responsible for the retention of wall morphology or Neurospora crassa after enzymatic attack. In other cases, melanin has been reported to be responsible for resistance of fungal cells to enzyme lysis and is thought to be involved in cell integrity. However, Bull (1970a) suggested that components other than melanin were involved in the prevention of total wall dissolution. He found that a resistant "core" of chitin and β -glucan persisted after enzymatic lysis of Aspergillus nidulans walls. In contrast to this, Katz and Rosenberger (1970) reported that normal chitin synthesis was not a factor influencing shape, and that the osmotic stress had a profound effect on wall morphology. Mutants of Aspergillus nidulans producing hyphae that lack chitin still maintained their characteristic shape and formed normal branches, provided the concentration of stabilizer was sufficiently high. In I. mentagrophytes microconidia, wall residues retained their characteristic shape after removal of the outer wall (OW1 or OW2) provided the inner wall remained intact (Fig. 13c). Even when walls were reduced to a thin, double-layered outer wall, by stripping off the inner wall with glucylase (Fig. 20b-c), the overall shape and rigidity was preserved when the rodlets were intact. This shape was lost only when the rodlet layer also disappeared. (Fig. 20d). These data indicate that the disintegration of microconidial wall only occurred following enzymatic or chemical treatments by sequentially attacking all the principle wall

components, and that the integrity of the microconidial wall does not reside in any one major wall component.

T. mentagrophytes causes several types of "tinea" in different parts of the body. For example, "athlete's foot" or "tinea pedis" is one of the most common diseases caused by this dermatophyte. Although it is known that the vegetative hyphae of this dermatophyte are responsible for the erosion and penetration of the infected tissues, the role of the abundantly formed microconidia in the pathogenicity and epidemiology of dermatomycoses is less well understood.

The identification of the various wall layers present in T. mentagrophytes microconidia, along with their chemical nature, as revealed by this study, is significant biologically. The roles of individual wall polymers involved in various structures and functions of the walls can be studied. The maintenance of the rigidity and shape of the wall can be elucidated through the study of mutants unable to make a specific wall component. By comparing the differences between the walls of mutants and those of the normal strains, the changes induced by the mutation can be revealed. This in turn may explain how the absence of a certain polymer or component influences the wall assembly. Since microconidia give rise to the vegetative hyphae which are infectious, the involvement of various wall polymers during the process of germination or conidiation will be of significance for the elucidation of mechanisms involved.

SUMMARY

Microconidial walls of Trichophyton mentagrophytes were isolated in high purity free from cytoplasmic contamination. The microconidial wall as seen in thin section was composed of an outer electron dense layer (25-50 nm) and a thicker inner layer (250-300 nm) of light electron density. The outer layer could be further resolved into two electron dense layers (OW1 and OW2) sandwiching a less electron dense middle layer. The shadowed wall fragments showed the outer surface to be amorphous, while the inner surface was sparsely covered with microfibrils. Electron microscopy of the freeze-etched surface of the microconidial wall revealed the presence of a crystalline rodlet layer made up of short fibers oriented in juxtaposition. To our knowledge, this has not been reported in any dermatophyte spores.

Chemical and enzymatic analyses of the microconidial walls showed the presence of carbohydrate (79.4 %), protein (22.6 %), lipid (3.5 %), ash (1.7 %), and trace amounts of phosphorus (0.2 %). Galactose, glucose and mannose (trace : 3 : 1) were detected in wall hydrolysates, while glucosamine was the only amino sugar identified.

The extraction and fractionation procedure devised in our laboratory permitted the separation of the microconidial wall into four principal fractions, which were subjected to quantitative and qualitative analyses.

Extraction of microconidial walls with 0.1 M sodium phosphate buffer (pH 6.5) containing 8 M urea-1 % mercaptoethanol-1 % SDS at room temperature for 8 hr (hereafter abbreviated as "UMS extraction"), preferentially

removed the bulk portion of the outer electron dense wall layer (OW1) (ca. 30-32 % of intact wall dry weight). The UMS soluble fraction (F-I), was composed of a homogeneous protein-carbohydrate-lipid complex. Paper chromatography of the acid hydrolysate revealed the presence of glucose and mannose at a ratio of 2:1. Only trace amounts of galactose were detected. Amino acid analysis of this fraction disclosed the common amino acids usually found in protein.

Subsequent extraction of the UMS treated walls with 1 N NaOH at 100 C for 1 hr solubilized the rodlet layer present on the microconidial surface. In thin section, one of the outer electron dense layers (OW2) of the UMS extracted walls was completely removed. This fraction (F-II) showed the presence of glucose and mannose (1:1), and protein. The alkali-insoluble residue (31.6 % of the intact walls) was shown to be composed of (1→3)-β-D-glucan (55.6 %), chitin (39.7 %) and protein (4 %).

Further treatment of the UMS and hot alkali treated wall residue (R-II) with 1 N H₂SO₄ at 100 C for 1 hr removed most of the cementing glucan matrix. Extensive acid hydrolysis (1 N H₂SO₄ at 110 C for 16 hr) removed completely the glucan matrix causing dissolution of some chitin fibers. These walls no longer retained their structural identities.

Treatment of intact walls with snail digestive enzymes (glucanase) completely degraded the inner wall layer leaving a residue containing the two electron dense outer layers (OW1 and OW2). This residue still retained the rodlet layer on its surface when examined

with the freeze-etching technique. Further treatment of this layer with hot alkali (1 N NaOH, 100 C for 1 hr) resulted in its complete disintegration.

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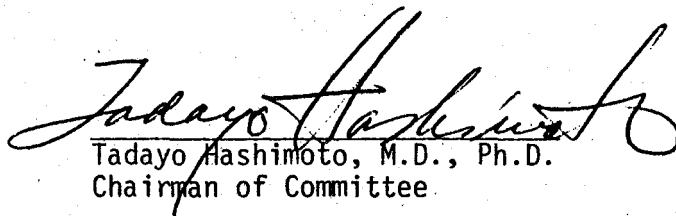
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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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